A study of the anti-androgenic effects of the phthalate ester, di-n-butyl phthalate, on two freshwater fish species, the fathead minnow and the three-spined stickleback.

A thesis submitted for the degree of Doctor of Philosophy

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Abstract

For the past few years there has been increasing concern surrounding a group of chemicals known as phthalate esters. In mammals, phthalates are known anti-androgens, interfering with the production or activity of testosterone. Phthalates are ubiquitous in the aquatic environment. With recent findings suggesting that anti-androgens may be responsible for much of the endocrine disruption found in wild fish populations, the study of phthalate esters has become integral to determining whether or not these anti-androgenic chemicals are of concern.

I investigated whether di-*n*-butyl phthalate (DBP) was able to cause anti-androgenic endocrine disruption in fish under controlled laboratory conditions. Three experiments were undertaken. In the first study, two generations of fathead minnows were exposed to nominal concentrations of 6 to 100 μg/L for 21 and 150 days, respectively. The second experiment examined the effects of early life-stage exposure to DBP (50, 100 and 200 μg DBP/L) on three-spined sticklebacks. The final experiment examined the effects of DBP on adult male three-spined sticklebacks in a 21-day nesting study (15 and 35 μg DBP/L).

DBP had no effect on the fecundity, survival, growth, sex ratio, or gonadal histology of the exposed fish in any of the experiments. Further, it failed to alter the expression of two steroidogenic genes in adult male sticklebacks. In contrast, DBP was often found to significantly alter plasma androgen concentrations in both species, and spiggin concentrations in the three-spined stickleback, most notably causing significantly reduced spiggin concentrations in the adult males exposed to DBP.

Ultimately, DBP-exposure did not disrupt the ability of the fish to reproduce successfully, and did not appear to alter reproductive behaviours or the expression of secondary sexual characteristics. In conclusion, while DBP did appear to have some capacity for endocrine disruption in fish, it was unable to interfere with the ability of the fish to develop normally and reproduce successfully. Thus, environmentally-relevant concentrations of phthalate esters are likely not of particular concern to fish populations.

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Declaration

The work described in this thesis was carried out between January 2006 and September 2009 at the Institute for the Environment, Brunel University, Middlesex, United Kingdom. The work was conducted independently, under the supervision of Prof. John Sumpter and Dr. Catherine Harris. It has not been submitted for any other degree.

Chapter 1. Introduction

1.1. Endocrine disruption in the environment

Ecotoxicology has been a field of interest to biologists since the late 1800s. The earliest reports documented effects in wildlife such as lead poisoning in pheasants after ingesting spent shot, and die offs of waterfowl following oil spills. With the invention and increased use of pesticides such as DDT in the late 1930s, the field of ecotoxicology expanded; chemicals were now found to not only persist in the environment, but also to cause unexpected effects in wildlife (Rattner 2009). Hundreds of examples of the effects of chemical use on the environment have arisen subsequently, from the threat of DDT to predatory bird populations, to the mortality in vultures caused by the consumption of cows treated with diclofenac (Moore and Walker 1963; Oaks *et al.* 2004; Rattner 2009). More recently, ecotoxicologists have become increasingly interested in the study of endocrine (hormone) disruptors and their effects in both humans and animals.

Endocrine disrupting chemicals (EDCs) comprise a vast range of substances. These chemicals elicit a wide array of effects and even more ways in which they induce such effects. Most endocrine disruptors studied to date alter the function of the two main vertebrate sex hormones, androgens and oestrogens. However, other steroidal hormones and peptide hormones may also be affected by potential EDCs (Sumpter 2005).

In terms of the sex hormones, endocrine disruptors tend to either be androgen or oestrogen agonists or antagonists. The mechanisms by which many endocrine disruptors function often centres on receptor binding. The receptors in question are mainly the androgen or oestrogen receptors, but other receptors such as the retinoid X receptor, and peroxisome proliferating-activated receptors can be involved

(Sumpter 2009). Generally, hormone agonists bind to the androgen or oestrogen receptors and, therefore, often have a similar structure to that of the hormone in question (Tyler *et al.* 1998). Endocrine antagonists, on the other hand, tend to have more variable mechanisms by which they interfere with endocrine systems. Some antagonists interfere with hormone-receptor binding, others decrease the affinity for or the number of receptors. Others interfere with hormone production by altering gene or enzyme expression, or with the availability of hormone precursors such as cholesterol (Stahlschmidt-Allner *et al.* 1997; Tyler *et al.* 1998).

Endocrine disruptors include several chemicals that were designed with the intention to specifically alter hormones in humans and animals, but also include a myriad of chemicals with unexpected endocrine disrupting activity, known as xenobiotics. Pharmaceuticals that are environmental endocrine disruptors include oestrogens such as the natural oestrogens, oestrone and 17β-oestradiol, and the synthetic oestrogen, 17α-ethinyloestradiol, found in the oral contraceptive pill (Sumpter and Johnson 2008). Pharmaceuticals can also be hormone antagonists. The drugs flutamide and dutasteride are both androgen antagonists, for example. Flutamide inhibits androgen-receptor binding (Ankley *et al.* 2004), while dutasteride is an α-reductase inhibitor, interfering with the production of the potent androgen dihydrotestosterone from testosterone (L. Margiotta-Cassaluci, personal communication). Such pharmaceuticals tend to be highly effective *in vivo* as they are designed specifically to elicit an acute response.

On the other hand, xenobiotics tend to be, but are not exclusively, much less potent. These EDCs come from a vast range of chemicals including PCBs, biocides and pesticides such as tribuytltin and DDT, surfactants such as alkylphenols, and plasticizers like phthalate esters (Stahlschmidt-Allner *et al.* 1997). Such chemicals also tend to be less specific in their activity, often with several mechanisms of action. For example, the pesticide DDT has several degradation products, some of which are oestrogen or androgen mimics and others which are anti-androgens, all with varying potency (Tyler *et al*. 1998).

With so many chemicals possessing the ability to disrupt hormone systems, it is not surprising that EDCs have been observed to affect a wide range of wildlife (Tyler *et al.* 1998). One of the earliest cases of EDCs affecting organisms in the environment was molluscs exposed to the pesticide, tributyltin. Beginning in the 1970s, many reports of female neogastropod snails with rudimentary penis outgrowths were published, but the cause of such changes remained unknown for several years. In 1981, the link between these effects and tributyltin was found (Smith 1981). This compound which was used in anti-fouling paint on the hulls of ships, was originally thought to be a highly potent anti-oestrogenic aromatase inhibitor causing increased androgen levels in females (Gibbs and Bryan 1996). Later it was determined that these organotins are highly potent agonists for the retinoid X receptor which induces male sexual development (Nishikawa *et al.* 2004).

Another example of endocrine disruption was reported in alligators in 1994. Eggs were collected from a highly polluted lake and an unpolluted control lake in Florida in 1992. The eggs were reared at a temperature known to yield a 1:1 sex ratio. The hatchlings were raised until 6 months of age, when they were sampled for blood and gonadal histology. The authors found significantly elevated plasma oestradiol concentrations in females and significantly lower plasma testosterone concentrations in males from the polluted site compared to control females and males, respectively. Histological analysis revealed a 100% incidence of polynuclear follicles (follicles containing more than one oocyte nucleus) in the females from the polluted lake, and poorly organized seminiferous tubules and abnormal cell morphology in males, compared to controls. Further, several males had smaller than normal phalli, with some females exhibiting the presence of a penis (Guillette *et al.* 1994).

In general, the study of endocrine disruption in the environment has centred mainly on fish, some of the first evidence of which was discovered in the UK. In 1978, a low incidence (~5%) of intersex and hermaphroditism was first discovered in wild roach (*Rutilus rutilus*) living downstream of sewage treatment plants on the River Lea (Sweeting 1981). However, this did not become widely known until male rainbow trout (*Oncorhynchus mykiss*) caged downstream from another sewage treatment plant outlet were found to be producing the egg yolk precursor protein, vitellogenin (VTG) (Purdom *et al.* 1994; Sumpter and Johnson 2008). Subsequent studies have demonstrated a widespread phenomenon of feminizing endocrine disruption in fish exposed to sewage effluent (Jobling *et al.* 1998).

While feminization has been the predominant effect observed in fish populations sampled in the wild, masculinization has also been noted, most incidences of which have been reported in association with exposure to pulp mill effluents (Larsson and Forlin 2002). However, this phenomenon may be more widespread. For example, a very high rate (14%) of spermatogenic tissue was observed in the ovaries of female pike (*Esox lucius*) in rivers across the UK, sampled both up- and downstream from sewage treatment plants (Vine *et al.* 2005).

Overall, the causative agents of endocrine disruption, in many cases, remain uncertain and likely vary with location (Sumpter and Johnson 2008). It is generally held that endocrine disruption in fish and wildlife results from exposure to a mixture of chemicals possessing various endocrine activities and mechanisms of action

(Hotchkiss *et al.* 2008). Surface waters tend to include a cocktail of oestrogenic and androgenic agonists and antagonists acting through various mechanisms of action and possessing a wide range of potency (Christiansen *et al.* 2002; Zafra-Gomez *et al.* 2008). In many cases, the main culprits for the feminization of fish are thought to be the synthetic and natural oestrogens oestradiol, oestrone and 17α-ethinyloestradiol (Desbrow *et al.* 1998), although xenoestrogens such as alkylphenols may also be involved (Sumpter and Johnson 2008). More recently, anti-androgens identified in surface waters have been hypothesized to be significant contributors to the incidence of intersex in fish (Jobling *et al.* 2009). Several chemicals identified in surface waters have been reported to have anti-androgenic activity *in vivo* and include the pesticide vinclozolin and the plasticizers, phthalate esters (Dargnat *et al.* 2009; Fatoki and Vernon 1990; Kolpin *et al.* 2002; Peijnenburg and Struijs 2006; Urbatzka 2007; Valsamaki *et al.* 2007). Therefore, it is possible that the intersex identified in wild roach caught downstream from sewage treatment plants reported by Jobling *et al.* (1998) may be the result of both oestrogenic as well as anti-androgenic pollution.

In order to understand the aetiology of endocrine disruption observed in the wild, ecotoxicologists must rely on robust and repeatable laboratory experiments to determine whether or not specific chemicals cause the effects observed in the environment. While not perfect, such experiments are able to tease out which chemicals possess endocrine disrupting activity in target organisms. In this manner, scientists can slowly uncover what risks such chemicals pose.

1.2. Phthalates

As the study of endocrine disruption in fish has shifted from a focus on estrogenic chemicals to the potential contribution of androgen antagonists, it has become increasingly important to identify which chemicals may be of concern. Among these anti-androgens, are a widely used group of chemicals known as phthalate esters.

Phthalate esters (or phthalates) were originally synthesized in the 1920s. They have a very large spectrum of uses in the production of pesticides, paper, household furniture, medications, perfumes, cosmetics and a number of other goods, but are most commonly used as plasticizers (Koch *et al.* 2005; Schettler 2006; Shen *et al.* 2007). In this application, phthalates are mixed with polyvinylchloride polymers, but they are not part of the plastic matrix. They impart flexibility to the plastic by lowering the glass transition point (Graham 1973). A significant proportion of the end product of a plastic may be phthalate: up to 40% of the final weight in some instances. As the phthalates are not chemically bound to the plastic, they leach out over time (Autian 1973). Phthalates are synthesized in very large amounts; globally, phthalate production is estimated at three million metric tonnes annually (Schettler 2006).

It has been estimated that in Europe, approximately 900,000 tonnes of phthalates are produced each year. Approximately two thirds of this are used in plastics. The remaining 300,000 tonnes make their way into various other applications such as personal care products (ECPI 2009). There are approximately eighteen industrially-important phthalate esters (Staples *et al.* 1997a). Of these, di (2-ethylhexyl) phthalate (DEHP) is the most widely used phthalate ester; butyl-benzyl phthalate (BBP) and di-n-butyl-phthalate (DBP) are also produced in high volumes (estimated at >20,000 tonnes per annum) (Harris *et al.* 1997).

The structure of phthalates is characterized by a benzene ring with two carboxylic acids attached in an ortho position (Figure 1.1). The carboxylic acids are alcohols with chain lengths of 1 to 13 carbons. Lipophilicity is an important determinant of the potency of phthalates in aquatic ecotoxicological studies, as it strongly affects their ability to bioconcentrate. For example, two of the most common phthalates, DEHP and DBP, have molecular weights of 390.6 and 278.4 g/mol respectively. Consequently, their water solubilities, as measured by the log octanol-water partition coefficient (logK_{ow}), are 7.50 and 4.45, with saturation points of 3 µg/L and 11.4 mg/L in water, respectively (Staples *et al.* 1997b). In general, studies have found that the widespread production and use of phthalates, combined with their level of solubility, makes them readily available to enter the environment. Once present, they mainly accumulate in organic phases such as soil and suspended particulate matter, but dissolve in water as well. Once present in surface waters, phthalates can readily bioconcentrate in fish (Staples *et al.* 1997b).

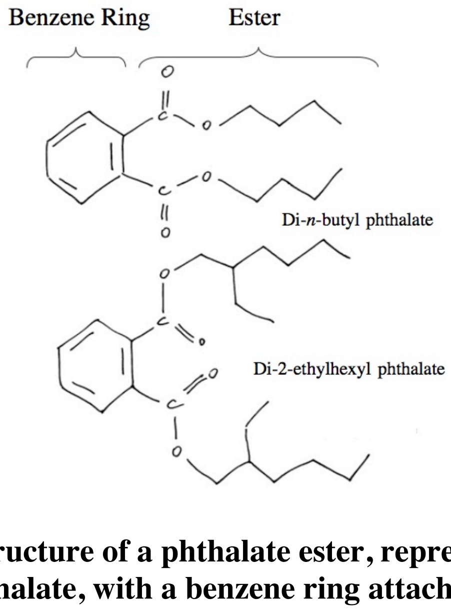


Figure 1.1. The basic structure of a phthalate ester, represented by di-*n*-butyl and di-2-ethylhexyl phthalate, with a benzene ring attached to two esters in an ortho position.

Phthalates are considered ubiquitous in the environment, and have been identified in soil, atmospheric, and aquatic samples worldwide (Fromme *et al.* 2002; Giam *et al.* 1980; Horn *et al.* 2004; Thomas 1973; Thuren and Larsson 1990). Phthalates have been detected in surface waters in Australia, Canada, the United States, Portugal, the Netherlands, France, the United Kingdom, China, Nigeria, and Malaysia, and have generally been measured in the low ng/L to µg/L range. At times, concentrations have been reported to reach tens to hundreds of µg/L, and in some cases, high mg/L concentrations (Cespedes *et al.* 2004; Fatoki and Noma 2002; Fatoki and Ogunfowokan 1993; Fatoki and Vernon 1990; Fromme *et al.* 2002; Furtmann 1994; Loraine and Pettigrove 2006; Mackintosh *et al.* 2006; Ogunfowokan *et al.* 2006; Peijnenburg and Struijs 2006; Tan *et al.* 2007; Tan 1995; Van der Velde *et al.* 1999; Vethaak *et al.* 2002; Wang *et al.* 2005) (Table 1.1 in Section 1.5.1). In addition to their detection in soil, air, and water, phthalates have been identified in stored blood at concentrations of 40-115 mg/L di-pentyl phthalate (DPP) and ~50 mg/L DEHP and in food up to 3.4 µg/g DEHP (Environment-Canada 1994a; Environment-Canada 1994b; Jaeger and Rubin 1972; Marcel and Noel 1970). These concentrations tend to be much higher than those reached in water because phthalates can easily leach from blood bags and food wrappings into the lipid-rich fractions of the blood (triglycerides and cholesterol), and food (fats and oils). In general, the median daily intakes of different phthalate esters in humans have been estimated at 0.3-33.9 µg/kg/day (Chen *et al.* 2008; Fromme *et al.* 2007; Koch *et al.* 2003).

Phthalates were, for many years, thought to be fairly benign substances, as early studies of acute toxicity showed very little potential for harm (Krauskopf 1973). Acute toxicology experiments revealed the dose lethal to 50 percent of the sample size (LD_{50}) ranged from 0.7 to over 20 g/kg in small rodents via intraperitoneal, intradermal, or oral routes (Gesler 1973; Krauskopf 1973). Inhalation is considered to be of little concern due to the low volatility of the chemicals, while oral intake is thought to be the most prominent route of exposure in humans (Schettler 2006). For several years chronic exposure of rats and mice to phthalates was considered to cause only general toxicological effects, and then only at very high doses (1-50 g/kg), affecting growth, kidney, liver and heart weights (Autian 1973). With adult No Observable Adverse Effect Levels (NOAELs) ranging from 40 to 330 mg/kg/day, it was concluded that phthalates were of little toxicological concern (Gesler 1973).

On the other hand, the potential of several phthalates to act as endocrine disruptors has been known for some time (Cater *et al.* 1977; Foster *et al.* 1982; Gray *et al.* 1977; Shaffer *et al.* 1945). Of the 18 commercially important phthalates, DEHP, DBP, BBP, di-*iso*-butyl phthalate (DIBP), di-*iso*-nonyl phthalate (DINP), di-hexyl phthalate (DHP), and DPP are now considered anti-androgenic endocrine disrupters in mammals (Foster *et al.* 1982; Foster *et al.* 1980; Gray *et al.* 2000; Gray *et al.* 1982; Staples *et al.* 1997a; Swan *et al.* 2005). Toxicity appears to be mainly related to the length of the ester groups, with 4-8 carbons being anti-androgenic (eg. DBP, BBP and DEHP), and less than 4 carbons producing benign phthalates (eg. di-ethyl phthalate (DEP) and di-methl phthalate (DMP)) (Foster *et al.* 1980; Foster *et al.* 1981; Howdeshell *et al.* 2008), although this has not been determined conclusively. From now on the term "phthalates" will, refer only to phthalate esters known to have anti-androgenic properties in mammals.

1.3. Effects of phthalate exposure in mammals

Phthalate-induced testicular damage has been observed in several rodent species including rats, guinea pigs, mice, and hamsters, exhibiting varying sensitivities between species (Gray *et al.* 1982). Anti-androgenic effects have also been noted in non-rodent species including the rabbit, boar, ferret and a small primate, the marmoset (Hallmark *et al.* 2006; Higuchi *et al.* 2003; Lake *et al.* 1976; Ljungvall *et al.* 2005)

It is generally held that males are more sensitive to the effects of phthalates than females (Lovekamp-Swan and Davis 2003; Wine *et al*. 1997). However, it should be noted that this does not negate the presence of effects in females. In fact, female rats appear to be affected at the same dose levels as males with (inconsistent) observations of delayed vaginal opening in females exposed *in utero* (Gray *et al*. 1999; Gray *et al*. 2006; Ma *et al*. 2006; Mylchreest *et al*. 1998). Females exposed as adults have been noted to have increased numbers of tertiary atretic follicles (Grande *et al*. 2006), pre-implantation loss (the loss of a fertilized embryo due to its failure to implant in the uterine lining), prolonged oestrous cycles (Davis *et al*. 1994), reduced plasma progesterone and oestradiol concentrations (Davis *et al*. 1994; Gray *et al*. 2006; Svechnikova *et al*. 2007), and a higher incidence of abortions at 500 mg DBP/kg/day, reaching 100% at 1000 mg/kg/day (Ema *et al*. 2000; Gray *et al*. 2006), However, not all reports are consistent, as elevated *ex vivo* oestrogen production in female rats has also been reported following *in vivo* phthalate exposure (Gray *et al*. 2006). Generally, it seems that females are largely ignored because the incidence of such effects is usually much lower (Wine *et al*. 1997).

In both sexes, sensitivity also appears to be inversely correlated to age. Neonates appear to be the most sensitive to adverse effects, followed by pubertal animals, with adults being the least sensitive (Gray and Butterworth 1980; Higuchi *et al*. 2003; Li *et al*. 2000; Li *et al*. 1998; Sjoberg *et al*. 1986; Wine *et al*. 1997).

1.3.1. Effects of phthalate exposure in adult versus neonatal mammals

The increased sensitivity of neonates is, on one hand, related to the fact that mammals exposed *in utero* appear to be affected at concentrations lower than those required to elicit effects in adults (Gray and Butterworth 1980; Higuchi *et al*. 2003; Li *et al*. 2000; Li *et al*. 1998; Sjoberg *et al*. 1986; Wine *et al*. 1997). Toxicology studies exposing rat dams to oral doses of various phthalates during pregnancy have shown that effects in offspring can be elicited at doses as low as 0.1 mg/kg/day (Lehmann *et al.* 2004). However, such findings are rare, and it is generally held that doses of 500 mg/kg/day *in utero* are required to cause abnormal development ranging from irreversible reproductive abnormalities to the complete loss of fertility in male offspring (Barlow *et al.* 2004; Barlow and Foster 2003; Culty *et al.* 2008; Kwack *et al.* 2009; Mylchreest *et al.* 1999; Mylchreest *et al.* 2002; Mylchreest *et al.* 2000).

However, the same dose levels can also induce significant changes in males exposed as adults, such as reduced testis weight, reduced sperm and dysgenic seminiferous tubules. In contrast to male rats exposed *in utero*, these changes are less severe and are also often reversible (Gray and Butterworth 1980; Gray *et al.* 1977; Kwack *et al.* 2009; Sjoberg *et al.* 1986; Wine *et al.* 1997). Therefore, it seems that the more severe effects observed in the phthalate-exposed neonates may be more a result of the timing of exposure. In other words, it would appear that in this case, phthalates can have drastic and irreversible consequences when administered during critical periods in development.

The increased sensitivity of neonates compared to adults was most clearly demonstrated in a multigenerational study conducted on rats exposed to 0.1, 0.5, and 1.0% DBP in the diet (equivalent to roughly 52, 256, and 509 mg/kg/day in males, and 80, 385, and 794 mg/kg/day in females). While no histological analysis was conducted, few effects were observed in F_0 males; sperm motility, concentration, and counts were normal. F_0 females were significantly lower in weight following birth of their litters compared to controls, but no effects on oestrous cycles were noted. Despite treatment for 14 weeks, the F_0 adults gave birth to 20 viable litters out of 20 at the highest dose level, although the number of live pups per litter was reduced in a statistically significant dose-dependent manner. By contrast, when the F_1 generation had matured (exposed *in utero* until termination), the highest dose level (~509-794 mg/kg/day) group were found to have significantly reduced rates of pregnancy, fertility, and mating, with only one live litter produced from 20 breeding pairs compared to 19 out of the 20 in controls. Such differences between F_0 and F_1 generations were attributed to the many morphological and histological abnormalities noted in the F_1 males, which are now considered characteristic of *in utero* phthalate exposure (Wine *et al.* 1997).

The findings of the multigenerational study, conducted more than a decade ago, fuelled further interest in the potential for anti-androgenic phthalates to act as reproductive toxicants. It is now widely accepted that when anti-androgenic phthalate esters, such as DBP, DEHP, BBP, and DINP are administered during the critical window (between gestation days (GD) 12-21), they induce a spectrum of effects in male rat offspring characteristic of *in utero* androgen disruption, and similar to “Testicular Dysgenesis Syndrome” in humans (Fisher *et al.* 2003; Foster

2006; Gray *et al.* 2000; Liu *et al.* 2005; Mylchreest *et al.* 1998; Mylchreest *et al.* 1999; Mylchreest *et al.* 2002; Mylchreest *et al.* 2000).

1.3.2. Effects of phthalate exposure in neonatal rats

The effects observed in rats following *in utero* exposure lie along a spectrum of severity corresponding to dose. In general, phthalates are fairly weak endocrine disruptors; most effects are noted at 100 mg/kg/day, and more commonly at doses of 500 mg/kg/day. At lower doses, significant differences in male rats exposed *in utero* include changes to steroidogenic gene expression at concentrations as low as 0.1 mg/kg/day (Lehmann *et al*. 2004), Leydig cell aggregation and increased testicular testosterone concentration at 10 mg/kg/day (Lin *et al*. 2008), delayed preputial separation (an androgen-dependent indicator of puberty) at 15 mg/kg/day, and even increased testis weight, observed to be significant at 5 mg/kg/day by (Andrade *et al*. 2006). However, changes are not consistently observed in male rats exposed to concentrations lower than 500 mg/kg/day (Mylchreest *et al*. 2002; Wine *et al*. 1997). Further, it is unknown whether the changes observed at lower doses are permanent and irreversible, as they are at higher doses (Barlow *et al*. 2004; Barlow and Foster 2003; Borch *et al*. 2004).

The more severe and irreversible effects of *in utero* phthalate exposure include changes to androgen-dependent structures in males, which often contribute to reduced fertility. These changes include the retention of nipples and reduced anogenital distance (the length of the perineum, which is normally longer in male mammals), underdevelopment/absence of accessory reproductive organs and epididymides, hypospadias (the opening of the urethra along the base of the penis), cryptorchidism (undescended testes), and dysgenic seminiferous tubule structure

(Andrade *et al.* 2006; Fisher *et al.* 2003; Gray *et al.* 2000; Mylchreest *et al.* 1998; Mylchreest *et al.* 1999; Mylchreest *et al.* 2002; Mylchreest *et al.* 2000; Thompson *et al.* 2005; Yamasaki *et al.* 2009). In some instances, although rare, high dose *in utero* phthalate exposure has even led to unilateral or complete agenesis of the testes in the male and of the uterine horns in the female (Gray *et al.* 1999; Gray *et al.* 2000).

1.3.2.1. Effects of phthalates on testosterone concentration and androgen-dependent structures

Testosterone is the most biologically important androgen synthesized in the mammalian testis, and is produced by the Leydig cells. Although testosterone may be converted into other hormones such as dihydrotestosterone or oestrogen, it is the major circulating androgen, responsible for the development and maintenance of the male sexual characteristics in the vertebrates (Stocco and McPhaul 2006). *In utero,* testosterone production begins at GD 15.5 in rats and continues after birth (Warren *et al.* 1973). It is essential to the male foetus for the differentiation of the gonads into testes and the internal sexual structures, and for the development of the external genitalia. In maturity, testosterone is responsible for the development of male secondary sexual characteristics, and the maintenance of spermatogenesis (Stocco and McPhaul 2006).

Reduced testosterone concentrations are one of the earliest and most sensitive effects of anti-androgenic phthalate exposure. Such changes are reversible, and while they are more commonly measured as testicular testosterone concentrations, they are often reduced in blood plasma as well (Akingbemi *et al.* 2001; Borch *et al.* 2004). It is generally accepted that immediately following phthalate exposure, testosterone levels decline in parallel with reduced expression of the steroidogenic genes responsible for testosterone synthesis and reduced mitochondrial uptake of cholesterol, a precursor of testosterone (Thompson *et al.* 2004). Steroid acute regulatory protein (StAR), scavenger receptor B1 (Scarb1 or Srb1), P450 side chain cleavage enzyme (Cyp11a1 or P450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD), and cytochrome P450c17 (Cyp17a1 or Cyp17) (but not 17β-hydroxysteroid dehydrogenase (17β-HSD)) are all significantly downregulated in the testes following phthalate exposure (Barlow *et al.* 2003; Borch *et al.* 2004; Lehmann *et al.* 2004; Lin *et al.* 2008; Shultz *et al.* 2001; Struve *et al.* 2009). Such changes have been observed at concentrations as low as 50 mg/kg/day in rats on GD 19 exposed *in utero* (Lehmann *et al.* 2004).

However, several publications have reported contradictory findings. In some cases, exposure of mammals to lower phthalate doses has been found to result in increased testicular and/or plasma androgen concentrations compared to controls, while at higher doses they are found to be significantly decreased (Akingbemi *et al.* 2004; Akingbemi *et al.* 2001; Lin *et al.* 1995). Further, two reports of upregulation of StAR, SR-B1, Cyp17 and downregulation of Cyp19 were published from the same laboratory following prepubertal exposure of males to ≥500 mg/kg/day of DEHP or DBP, despite decreased plasma testosterone concentrations (Lee *et al.* 2009; Ryu *et al.* 2007). Another study found increased expression in StAR and Cyp17 at 10 and 100 mg MEHP/kg/day following 12 hours exposure in rats at post-natal day (PND) 28, although in a manner independent of dose (Lahousse *et al.* 2006). Finally, a study exposing male rats to DEHP *in utero* at concentrations from 117-1250 mg/kg/day found that while *ex vivo* testosterone production in the neonatal males was significantly reduced, gene expression levels of P450scc, P450c17, and StAR were often increased, even though plasma testosterone concentrations remained significantly low into adulthood (PND 60) (Culty *et al.* 2008). Hence, there is still some uncertainty surrounding the mechanism behind the reduced androgen concentration.

Regardless of the molecular basis for the effect, reduced testosterone synthesis caused by phthalates during critical periods in foetal development is the most likely cause of malformation or absence of several testosterone-dependent structures. This includes malformations of the prostate, Wolffian Duct System, and external genitalia (eg. hypospadias), the retention of nipples, a shorter perineum resulting in reduced anogenital distance, and, most severely, the uni- or bilateral absence of the testes (Barlow and Foster 2003; Foster 2006; Howdeshell *et al.* 2007).

1.3.3. Histological effects of phthalates in mammals

1.3.3.1. Background

Examination of testicular cell structure following phthalate exposure shows that phthalates also have effects at the cellular level. Mammalian testes can be divided functionally into two areas: the seminiferous tubules, which are essential to spermatogenesis, and the interstitial area, which is responsible for endocrine function. The seminiferous tubules comprise the spermatogenic tissue of the testes, and consist of two main types of cells, the Sertoli cells and the germ cells, the latter of which develop into sperm. The interstitial area surrounds the seminiferous tubules and contains the blood vessels, lymphatic system, nerve fibres, and testosterone-producing Leydig cells (Figure 1.2).

Sertoli cells are central to the development and functioning of the testes. They have a dual role in male reproduction: first, in the development of the testes, and second, in supporting spermatogenesis. The Sertoli cells are the first cells to differentiate in the rudimentary vertebrate sex cords. They enable the development of the seminiferous cords, prevent germ cell entry into meiosis, and promote the differentiation and function of other somatic cells, such as the Leydig cells. Later on, the Sertoli cells take on a spermatogenic role. The process of spermatogenesis is heavily reliant on Sertoli cells, which provide a highly structured and specialized environment for development of the germ cells both structurally, within their numerous lateral folds, and chemically, via the blood-testis barrier (Griswold and McLean 2006; Sharpe *et al*. 2003)(Figure 1.2).

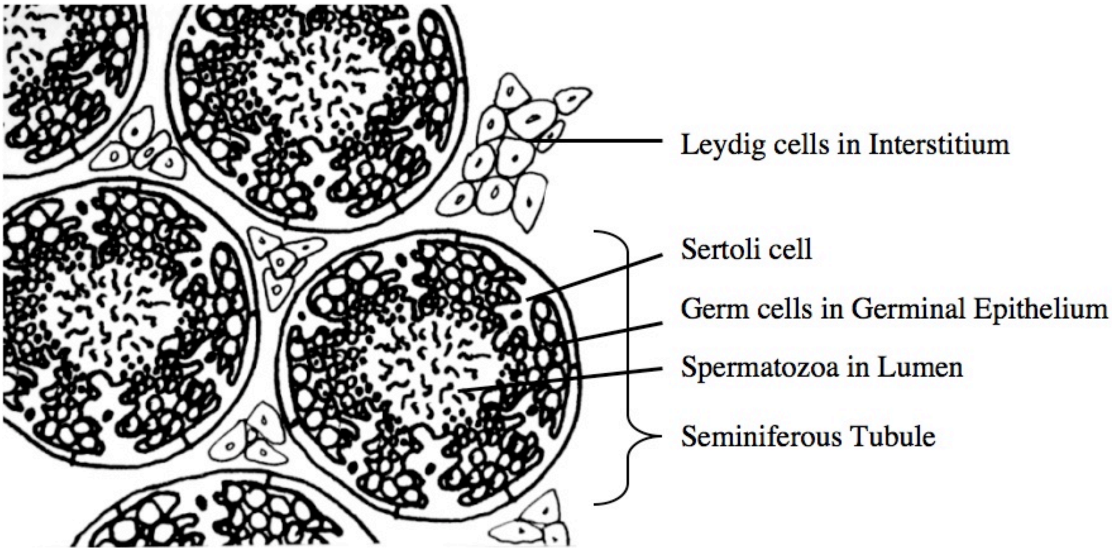


Figure 1.2. Depiction of a mammalian testis in cross section, showing several seminiferous tubules containing developing sperm cells and spermatozoa, the seminiferous tubules are surrounded by Leydig cells in the interstitial space.

A germ cell is in contact with a single Sertoli cell throughout its development, moving gradually from the distal regions of the seminiferous tubule towards the lumen. During this development, the Sertoli cell is constantly changing its structure to accommodate the differentiating germ cells. This structure is partly reliant on the vimentin cytoskeleton within the Sertoli cell, and its collapse has been noted to result in germ cell apoptosis, underlining the importance of the relationship between the two cell types (Show *et al.* 2003). Due to this specific interaction, each Sertoli cell can only support a fixed number of germ cells. There is therefore a direct correlation between the number of Sertoli cells and the spermatogenic capacity of a male (Orth *et al.* 1988).

Sertoli cell proliferation is therefore an important determinant of fertility. In humans it occurs at two discrete time points in development, during the fetal period and at puberty. In rats, Sertoli cell proliferation spans GD 15.5 to PND 21 (Orth 1982). It is induced mainly by FSH, but is also affected by non-hormonal growth factors. The number of Sertoli cells, and thus spermatogenic capacity, is fixed thereafter (Griswold and McLean 2006; Sharpe *et al.* 2003).

The Leydig cells are the other main somatic cell essential to normal reproduction in the vertebrate male. As previously stated, Leydig cells are responsible for the production of testosterone, and are induced by luteinizing hormone (LH). Leydig cells surround seminiferous tubules in the interstitial space, forming small clusters (Figure 1.2). Their differentiation is first induced by LH, but is also dependent on Sertoli cell paracrine signalling (from cell to cell) (Kerr *et al.* 2003; Yao *et al.* 2002).

Phthalates appear to have drastic effects on the histology of the developing testes, affecting the seminiferous tubules at doses of $\geq$135 mg DEHP/kg/day and Leydig cells at higher concentrations (~500 mg DBP/kg/day) (Andrade *et al.* 2006; Barlow and Foster 2003). Effects include severe degeneration and atrophy of the tubules, changes in Sertoli cell structure and proliferation, disrupted Sertoli-germ cell interactions, reduced spermatogenesis, and effects on Leydig cell size, aggregation, and/or hyperplasia (Mylchreest *et al.* 1998).

1.3.3.2. Seminiferous tubule effects

Changes to the seminiferous tubules are somewhat sensitive to phthalate exposure (~135 mg/kg/day), and in some instances these are permanent, persisting into adulthood (PND 540) (Andrade *et al*. 2006; Barlow *et al*. 2004; Mylchreest *et al*. 1998). Effects on seminiferous tubules, as previously stated, include changes to the architecture of the tubule, degeneration of the germinal epithelium, and changes to cell morphology (Andrade *et al*. 2006; Gray *et al*. 2000; Kleymenova *et al*. 2005; Mylchreest *et al*. 2000). Convolution and dilation of the seminiferous tubules has been noted in males exposed *in utero,* persisiting to 540 PND following 500 mg/kg/day but not 100 mg/kg/day DBP exposure (Barlow *et al*. 2004). Generally, dysgenesis following high-dose *in utero* phthalate exposure is fairly persistent and prevalent, with degeneration of the seminiferous epithelium noted in 43 to 100% of mature rats at maturation (PND 90-110) ($\geq$500 mg DBP/kg/day) (Fisher *et al*. 2003; Mylchreest *et al*. 1998; Mylchreest *et al*. 2000).

The effects of phthalates on the cells within the seminiferous tubules are some of the earliest observable effects following exposure *in utero* and include three major changes: 1. non-permanent alteration of Sertoli cell ultrastructure, 2. abnormal germ cell morphology, including multinucleated gonocytes, 3. loss of germ cells or Sertoli cell-only tubules.

Changes to Sertoli cell ultrastructure have been noted within as little as three hours of administration of a single, albeit high, oral dose of phthalate to adult male rats (2.2 g DBP/kg). Sertoli cells have been observed to have decreased cytoplasmic density, increased mitochondrial size, and reduced lateral processes (Creasy *et al.* 1987). In rats exposed *in utero*, observations are similar; Sertoli cells tend to become decreased in diameter, have reduced lateral processes and interaction with germ cells (on GD 19-21). However, such changes appear to revert to normal postnatally (Kleymenova *et al.* 2005).

Changes to germ cell morphology include increased numbers of germ cells with concomitant reduction in their size (Barlow and Foster 2003). Multinucleated gonocytes are spermatogenic cells that have divided improperly following meiosis and mitosis and are not viable. They are commonly observed in the dysgenic areas of phthalate-treated juvenile rat testes and have been noted following exposure to doses of phthalate as low as 100-135 mg DEHP/kg/day (Andrade *et al.* 2006; Boekelheide *et al.* 2009), but are generally observed at higher doses (~500 mg DBP/kg/day) (Barlow and Foster 2003; Ema *et al.* 1992; Fisher *et al.* 2003; Gray *et al.* 2000; Kleymenova *et al.* 2005; Mylchreest *et al.* 2000). Multinucleated gonocytes are thought to result from a lack of interaction with Sertoli cells, resulting in improper cell division. They are generally only observed during the foetal period beginning GD 16 until PND 10-16 (Fisher *et al.* 2003) and contain 2-4 nuclei. It is thought that eventually they apoptose (Barlow and Foster 2003).

Finally, decreased spermatogenesis and loss of all germ cells in dysgenic areas leading to Sertoli cell-only tubules, is generally observed at phthalate doses of approximately 500 mg DBP/kg/day or higher. This term, “Sertoli cell-only tubules” refers to tubules consisting of a layer of Sertoli cells surrounding an empty lumen (Figure 1.3) (Fisher *et al.* 2003). The degenerating germ cells are thought to first manifest as multinucleated gonocytes, which are thought to apoptose after PND 16. Anastomosed seminiferous tubules without germ cells then increase in frequency in late puberty (PND 90) and persists into adulthood (PND 540) (Barlow *et al.* 2004; Barlow and Foster 2003; Fisher *et al.* 2003; Mahood *et al.* 2005).

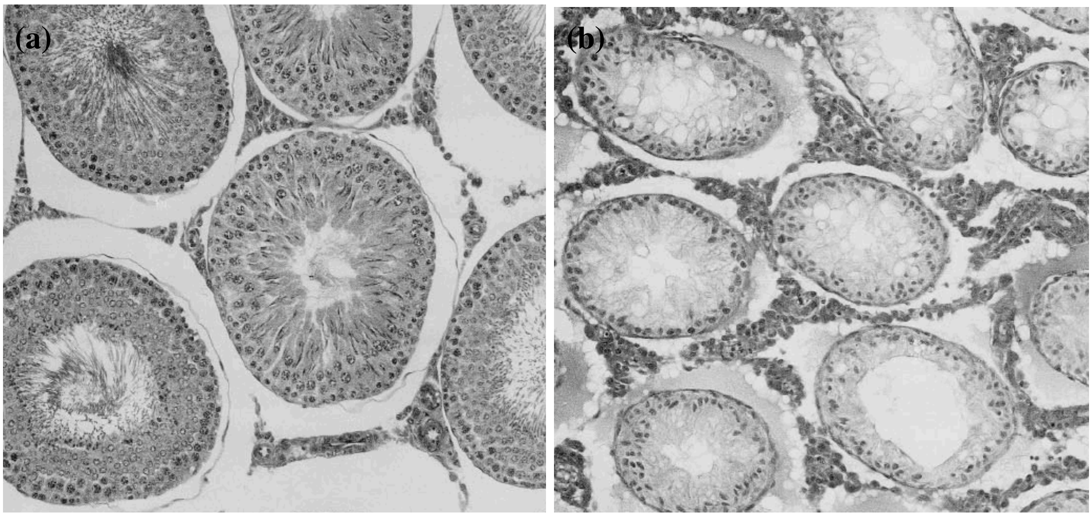


Figure 1.3. Transverse sections of the testes of rats from the (a) control, and (b) 500 mg DBP/kg/day groups exposed *in utero*. (a) This control testis has round seminiferous tubules with spermatozoa in the lumen. (b) The tubule lumens are empty due to the exfoliation of the germinal epithelium, and outside, larger clusters of Leydig cells are also apparent (Mylchreest *et al.* 1999).

Reduced sperm production has been frequently noted following exposure to high doses of phthtalate (≥500 mg/kg/day) (Higuchi *et al*. 2003; Lee *et al*. 2009; Sharpe *et al*. 1995) and is likely associated with a 100% loss of germ cells noted in dysgenic areas of the seminiferous tubules (Fisher *et al*. 2003). In general, it is hypothesized that multinucleated gonocytes, the loss of germ cells, and ultimately reduced spermatogenesis, are all results of disruption of Sertoli-germ cell interactions. This reduced interdigitation between germ and Sertoli cells has also been noted following juvenile and adult exposures to phthalates (Fisher *et al*. 2003; Kobayashi *et al*. 2003).

1.3.3.3. Leydig cell effects

Phthalate-induced changes in hormone-producing Leydig cells are observed following *in utero* exposure in male rats at phthalate doses as low as 10 mg/kg/day (Lin *et al*. 2008). Hyperplasia of Leydig cells has been reported as early as GD 16 with phthalate administration, and can be noted in the form of adenomas (benign tumors) in adulthood (PND 540) (Barlow *et al.* 2004; Barlow and Foster 2003). Hyperplastic Leydig cells are generally found alongside dysgenic seminiferous tubules and are described as small and spindloid with reduced, tightly packed cytoplasm (Barlow *et al.* 2004). It is still uncertain whether or not Leydig cell hyperplasia is due to increased proliferation of Leydig cells, as indicated by increased PCNA staining (Mylchreest *et al.* 2002), or whether the hyperplasia is instead a form of increased aggregation whereby clusters of Leydig cells simply comprise more and smaller cells than the controls (Boekelheide *et al.* 2009; Mahood *et al.* 2005).

1.3.4. Mechanisms of action of phthalates

Although the specific mechanism of action of phthalates as androgen antagonists has yet to be determined, it is widely accepted that the active compound of anti-androgenic phthalates is not the diester, but the primary metabolite, the monoester (Creasy *et al*. 1987; Foster *et al*. 1982; Gray and Beamand 1984). With one exception (Dr. Catherine Harris, Institute for the Environment, personal communication), anti-androgenic phthalates have not been found to interact with the androgen receptor (Foster *et al*. 2001; Parks *et al*. 2000; Sultan *et al*. 2001), so some other mechanism(s) is/are likely operative. The Leydig cells are not thought to be the cellular targets because direct effects on these cells *in vitro* have only been observed at concentrations at least four orders of magnitude higher than those required to disrupt Sertoli cells (1 mM versus 10^{-4} mM MEHP in primary cultures) (Jones *et al*. 1993; Li *et al*. 1998; Lloyd and Foster 1988). Further, a single *in vivo* exposure (2.2g DPP/kg) in rats was observed to affect Leydig cells only after effects had been observed in Sertoli cells (Creasy *et al*. 1987).

Proposed mechanisms of action include disruption of the follicle stimulating hormone-induced cAMP production pathway (Grande *et al*. 2006; Grasso *et al*. 1993; Heindel and Powell 1992), loss of zinc causing testicular dysgenesis (Cater *et al*. 1977; Foster *et al*. 1982; Foster *et al*. 1981; Oishi 1986; Oishi and Hiraga 1983; Peters *et al*. 1997), induction of Fas-signalling (Lee *et al*. 1999; Lee *et al*. 1997; Richburg 2000), action as a peroxisome proliferator (David *et al*. 2000; David 2000; Gazouli *et al*. 2002; Kobayashi *et al*. 2003; Ward *et al*. 1998), the inhibition of Sertoli cell proliferation (Li *et al*. 1998), and the inhibition of the conversion of glucose from glycogen causing germ cell apoptosis (Kuramori *et al*. 2009).

1.4. Human exposure

1.4.1. Testicular dysgenesis syndrome

It is widely believed that phthalate concentrations causing adverse effects in animal models in the laboratory are much higher than any estimated exposures occurring in humans. However, recent epidemiological studies have found significant correlations between concentrations of phthalate metabolites in blood and/or urine and testicular dysgenesis syndrome in the human population. These relationships occur at concentrations much lower than those known to be hazardous.

Testicular dysgenesis syndrome is a constellation of abnormalities in newborn and young adult human males thought to stem from abnormal testicular development *in utero*. Once thought to be discrete abnormalities, the syndrome includes reduced spermatogenesis, testicular cancer, hypospadias, and cryptorchidism (Skakkebaek 2004), and appears to be an increasing problem in males in the Western world (Virtanen *et al.* 2005). For example, testicular cancer and low and declining sperm quality are thought to be increasingly prevalent in cohorts of younger men (Bergstrom *et al.* 1996; Irvine 1996). Since there are so many similarities between the effects of phthalates in mammalian exposure studies and testicular dysgenesis syndrome, a causative link between the two would be an extremely important finding in the aetiology of reproductive disorders in the male population. However, the link has yet to be conclusively demonstrated.

1.4.2. Estimated concentrations of phthalate-exposure in the human population

Human exposure to phthalates, although difficult to ascertain with certainty, is generally considered to be in the range of 0 to 310 µg/kg/day (Chen *et al*. 2008; Fromme *et al*. 2007; Koch *et al*. 2003). To avoid the analytical problem of background contamination, many researchers undertake analysis of the major urinary metabolites of phthalates, which are readily broken down into primary and secondary metabolites. These metabolites are easily found in blood plasma, excreted mainly via the urine, and are good indicators for phthalate exposure (Wittassek and Angerer 2008).

Epidemiological studies of urinary metabolites of phthalates in the American population for the National Health and Nutrition Examination Survey (NHANES) conducted from 1988-1994 and again from 1999-2000 have shown that the anti-androgens MBP, and MBzP (from DBP and BBP, respectively), as well as mono-ethyl phthalate (MEP from DEP), can be detected in >97% of samples from adults and children, while MEHP (from DEHP) was detected with a ~75% prevalence. The incidence of other metabolites from less common phthalates, such as di-3-carboxypropyl phthalate (DCP), DINP, and di-octyl phthalate (DOP), tends to be lower ($\leq$16%) (Blount *et al.* 2000; Silva *et al.* 2004). Median concentrations of phthalate metabolites have ranged from low µg/L to several hundreds of µg/L in urine (for example, 2.7 µg MEHP/L, 21.2 µg MBP/L, 41 µg MBzP/L, 305 µg MEP/L) (Blount *et al.* 2000).

In a study of human phthalate exposure in a German population, MBP and MBzP metabolites were measured in urine samples from 36 children and 19 adults (median ages of 4.7 and 37.4, respectively). All samples had measurable concentrations of the two phthalate monoesters. Median MBP results for children were 139 compared to 91.8 µg/L in adults, whereas MBzP concentrations were much lower at 22.1 µg/L in children and 12.7 µg/L in adults (Koch *et al.* 2005).

Interestingly, the MBP concentrations were at least three times higher in the German population compared to the NHANES study. Possible explanations may be based on the differing phthalate exposures in these populations, but may also reflect analytical or time-related differences (Brock *et al.* 2002; Koch *et al.* 2005; Silva *et al.* 2004). Overall, exposure trends in the human population have shown higher concentrations of metabolites (other than MEP) in children compared to adults. Women also seem to have higher concentrations of MEP, MBP, and MBzP than men, whereas MEHP levels seem comparable between sexes. Likely explanations for age-dependent variation are a higher frequency of exposure, higher uptake to body mass ratio, and/or a less efficient rate of metabolism and excretion in children compared to adults. Higher concentrations in women may be due to more frequent use of personal care products containing phthalates, such as shampoos and cosmetics (Blount *et al.* 2000; Shen *et al.* 2007; Silva *et al.* 2004).

Humans working directly with phthalates have also been assessed and demonstrate the ease with which phthalates are taken up into human tissues.

Seventy-four exposed and 63 unexposed adult men working in the same polyvinylchloride manufacturing plant were examined in China in 2005. The concentration in the urine samples was normalised per gram of creatinine, and the urine samples of exposed workers had significantly higher concentrations of DBP and DEHP metabolites compared to those unexposed workers in the plant. Workers in direct contact with the phthalates had median concentrations of 548.4 µg MBP/g and 562.3 µg MEHP/g in their urine, while unexposed men had 113.4 µg MBP/g and 5.4 µg MEHP/g. Compared to American males in the NHANES survey, unexposed Chinese males had 7.5 and 2.0 times higher concentrations (µg/g creatinine) of MBP and MEHP, respectively. The daily phthalate intake of exposed Chinese men was estimated at 48.2 µg/kg/day, while unexposed Chinese men were estimated to be exposed to 0.5 µg phthalates/kg/day (Pan *et al.* 2006).

Collectively, these studies suggest phthalates are ubiquitous within the population and can be frequently measured at concentrations up to hundreds of µg/L in urine. To limit exposure, daily intake guidelines have been set by various governing bodies. The complexity of assessing daily exposures across human populations, combined with a lack of data on cumulative and/or synergistic responses to several phthalates, makes it difficult to set accurate limits. The European Union (EU) has used NOAEL data to derive the tolerable daily intake. For example, tolerable daily intake for DEHP is set at 50 µg/kg/day (based on a NOAEL of 5 mg/kg/day). Other tolerable daily intakes for established anti-androgenic phthalates are 150 µg DINP/kg/day, 850 µg BBP/kg/day, and 100 µg DBP/kg/day (CSTEE 1998). The US Environmental Protection Agency has also set oral tolerable daily intakes for several phthalates. In this instance, the limits are based on various mammalian studies undertaken from the 1950s to the 1980s, and have yet to be reviewed. Phthalates are considered to pose no risk to humans consuming less than 200, 100, and 20 μg/kg/day BBP, DBP, and DEHP, respectively (EPA 1991; EPA 1993a; EPA 1993b; EPA 1994).

1.4.3. Epidemiological evidence of a link between phthalates and testicular dysgenesis syndrome

An early study looking at the relationship between phthalates and testicular dysgenesis syndrome measured phthalate metabolite concentrations in human breast milk and their relationships to both plasma LH and testosterone concentrations and presence/absence of cryptorchidism in sons. The authors found significant positive correlations between phthalate levels in the mother's breast milk and the son's serum LH:testosterone ratios for MMP, MEP, and MBP. A significant positive correlation between milk MINP concentration and LH serum concentration in sons was also observed, as well as a significant negative correlation between milk MBP concentrations and the free testosterone in the serum of the sons, with similar non-significant effects for the other monoesters (n=96) (Main *et al.* 2006).

In another epidemiological study, concentrations of mono-*iso*-butyl, MEP, MBzP, and MBP metabolites in pregnant mothers, both individually and combined, significantly correlated with shorter anogenital index in their sons (median = 4.8 μg/L mono-iso-butyl phthalate, 225 μg/L mono-ethyl phthalate, 16.1 μg/L MBzP, and 24.5 μg/L MBP) (Swan *et al.* 2005). Interestingly, the concentrations measured in this study were similar, if not higher, than concentrations measured in the general female population surveyed by NHANES between 1999-2002 (NCEH 2005). The urinary phthalate concentrations of the pregnant women were converted to median estimated daily exposures of approximately 0.99 (DBP), 6.64 (DEP), 0.50 (BBP), and 1.32 µg/kg/day (DEHP) (Marsee *et al.* 2006). Although not directly comparable, these daily amounts are also 10-100 times lower than the tolerable daily intakes suggested by the US EPA and the EU to be unlikely to cause any deleterious effects.

Phthalate exposure has also been directly linked to reduced plasma testosterone levels in adult males. The men analyzed for phthalate exposure in a Chinese PVC factory had significantly reduced serum free-testosterone concentrations compared to unexposed men. LH, FSH and oestradiol concentrations were comparable. MBP alone, and MEHP combined with MBP, were significantly negatively correlated to serum testosterone concentrations in all workers collectively, and exposed workers alone, but not unexposed workers. Pan *et al.* (2006) estimated the daily exposure to phthalates at 48.2 µg/kg/day in males that worked directly with phthalates. This daily dose is, again, lower than the tolerable daily intake values set by the EU of 50 and 100 µg/kg/day for DEHP and DBP, respectively (CSTEE 1998), and provides evidence that these levels are much too high to prevent adverse effects in the human population (CERHR 2006; Pan *et al.* 2006).

These two studies provide strong support for the hypothesis that the phthalates able to induce the constellation of androgen-dependent abnormalities commonly observed in foetal male rats, are also causative agents of testicular dysgenesis syndrome in human male populations (Fisher *et al.* 2003; Sharpe 2005).

1.5. Phthalate esters and fish

1.5.1. Risks of phthalate esters to fish

The ubiquity of phthalate esters in surface waters has been well-documented worldwide (Cespedes *et al*. 2004; Dargnat *et al*. 2009; Fatoki and Noma 2002; Fatoki and Ogunfowokan 1993; Fatoki and Vernon 1990; Fromme *et al*. 2002; Furtmann

1994; Loraine and Pettigrove 2006; Mackintosh *et al.* 2006; Ogunfowokan *et al.* 2006; Peijnenburg and Struijs 2006; Tan *et al.* 2007; Tan 1995; Van der Velde *et al.* 1999; Vethaak *et al.* 2002; Wang *et al.* 2005). Phthalates can be readily detected in both fresh and marine surface waters. Concentrations of phthalates remain relatively low. Generally, they are measured in the ng/L to the low ug/L range; however, they have been measured in mg/L concentrations (Table 1.1).

With the ubiquity of phthalates in the aquatic environment established and the wide range of evidence supporting the ability of these chemicals to disrupt both human and mammalian endocrine systems, it seems highly likely that phthalate esters pose a potential risk to fish. In fact, fish may be more susceptible to endocrine disruption by phthalates than mammals due to the lipophilic properties of these chemicals coupled with their unique physiology. The usual route of mammalian exposure to EDCs is likely to be oral (Schettler 2006). The majority of ingested chemicals are metabolised first in the stomach by digestive enzymes, they are then transported to the liver via the hepatic portal vein, where they are metabolised further. It is only after these processes that the chemicals enter the bloodstream to reach their target tissues. By contrast, fish readily absorb lipophilic chemicals from the water via the gills. The gills are highly vascular structures, able to easily take up many hydrophobic chemicals directly into the bloodstream, which transports them to their target tissues. This is particularly important, since they bypass degradation and metabolism by the liver. Eventually, metabolism and excretion are likely fates for most EDCs in fish, but bioconcentration, and bioaccumulation also occur.

For reference, bioconcentration is the absorption of chemicals from water, resulting in higher concentrations in the tissues than in the surrounding water. This is due to the lipophilicity of chemicals which pass easily across the gill epithelium into the more lipophilic blood. By contrast, bioaccumulation is the accumulation of chemicals in tissues of animals from any source, including aqueous and dietary routes. In both cases, the chemicals accumulate in the tissues since the rate at which they are metabolized and excreted is slower than the rate at which they are taken up. Finally, biomagnification is when chemicals are found at higher tissue concentrations up trophic levels because these chemicals are not metabolized, but rather sequestered in tissues and accumulate through consumption. Popular examples of biomagnification include PCBs in arctic ecosystems and methylmercury in aquatic food webs (Fisk *et al.* 2005).

Bioconcentration factors (BCFs) for phthalates (the ratio of concentration of a chemical in tissue versus the water) have been reported to range from 10 to >2000 depending on initial concentrations, static or flow-through conditions, fish species and methods of analysis (Call *et al.* 1983; Mayer and Sanders 1973; Staples *et al.* 1997a; Staples *et al.* 1997b). Biomagnification does not appear to be a fate for phthalates in fish (Mackintosh *et al.* 2004; Norman *et al.* 2007). EDCs that 1) can persist in surface waters, 2) are readily absorbed by fish, and 3) are able to bioconcentrate and/or bioaccumulate, provide the circumstances for aquatic endocrine disruption in fish. However, we cannot assume that phthalates will automatically cause similar effects in fish as they do in mammals without some prior knowledge of the endocrine systems of both classes.

Table 1.1 . Reported concentrations of various phthalate esters in surface water samples and sewage treatment effluents across the world, in chronological order by the year of publication.

Year Sampled	Location	Water Source	Phthalate	Concentration Range	Mean	Median	Reference
1984	Rivers in Manchester, UK	Freshwater	DBP		21.5 μg/L		(Fatoki and Vernon 1990)
1990-91	SW Nigeria	Freshwater	DBP	<LOD – 1472 mg/L			(Fatoki and Ogunfowokan 1993)
			DEP	<LOD – 538 mg/L			
			DMP	<LOD – 462 mg/L			
1992-3	Klang River Basin, Malaysia	Freshwater	DBP	0.8-3.2 μg/L			(Tan 1995)
			DEHP	3.1-64.3 μg/L			
Unknown	Coastal South Africa	Salt and Freshwater	DMP	<LOD – 350 μg/L			(Fatoki and Noma 2002)
			DEP	<LOD – 398.3 μg/L			
			DBP	<LOD – 1028.1 μg/L			
			DEHP	2.1– 2306.8 μg/L			
1997	Four rivers across Germany	Freshwater	DBP	0.12-8.8 μg/L		0.50 μg/L	(Fromme *et al*. 2002)
			DEHP	0.33-97.8 μg/L			
1999	The Netherlands	Freshwater	DMP	<LOD-190 ng/L		17 ng/L	(Vethaak et al. 2005)
			DEP	<LOD-2300 ng/L		430 ng/L	
			DBP	<LOD-3100 ng/L		250 ng/L	
			DPP	<LOD-8 ng/L		6 ng/L	
			BBP	<LOD-1800 ng/L		77 ng/L	
			DMPP	50-2400 ng/L		380 ng/L	
			DEHP	<LOD-5000 ng/L		320 ng/L	
			DOP	<LOD-78 ng/L		15 ng/L	
2003-2004	Tanjin, China	Effluent	DBP	<LOD – 8.1 ng/L			(Wang *et al*. 2005)
			DEHP	1-23.8 μg/L			
			DIBP	<LOD – 14.2 ng/L			

Table 1.1. (continued)

Year Sampled	Location	Water Source	Phthalate	Concentration Range	Mean	Median	Reference
2001	Ter Basin, NE Spain	Freshwater	DBP	<LOD – 2.36 μg/L			(Cespedes *et al*. 2006)
			DEP	<LOD – 16.2 μg/L			
Unknown	British Columbia, Canada	Saltwater	DBP	50-244 ng/L	110 ng/L		(Mackintosh *et al*. 2006)
			DEHP	170-444 ng/L	275 ng/L		
2002 - 2003	Nigeria	Freshwater downstream from a sewage lagoon.	DMP	<LOD – 21.03 mg/L	10.41 mg/L		(Ogunfowokan *et al*. 2006)
			DEP	<LOD - 6.55 mg/L	6.18 mg/L		
			DPP	<LOD – 30.26 mg/L	18.67 mg/L		
			DBP	<LOD – 53.81 mg/L	19.44 mg/L		
			DEHP	17.82 – 80.53 mg/L	80.53 mg/L		
			DOP	<LOD	<LOD		
			DINP	<LOD	<LOD		
2001-2002	Southern California	Sewage Effluent	DMP	-	2.36 μg/L	0.045 μg/L	(Loraine and Pettigrove 2006)
			DEP	-	2.10 μg/L	0.97 μg/L	
			DBP	-	3.71 μg/L	2.70 μg/L	
			BBP	-	0.651 μg/L	0.26 μg/L	
			DEHP	4.49-20.7 μg/L	10.8 μg/L	3.53 μg/L	
2005-2006	Seine River Estuary, France	Freshwater	DEP	71-181 ng/L			(Dargnat *et al*. 2009)
			DBP	67-319 ng/L			
			DEHP	160-970 ng/L			

<LOD = below limit of detection of the assay.

1.5.2. Fish sensitivity to endocrine disruption versus mammal sensitivity

Major differences between teleostean and mammalian reproductive systems exist, including differences in gonadal structures, egg types, secondary sexual characteristics, and behaviours. However, the underlying endocrine system within all vertebrates is highly conserved.

The vertebrate reproductive system operates under the control of the hypothalamus-pituitary-gonadal axis. The peptide and protein hormones released by the hypothalamus and pituitary are chemically analogous, but not structurally identical throughout the vertebrate class. By contrast, the sex hormones are structurally identical throughout the class. Some differences do, however, exist. For example, testosterone is the main androgen in mammalian males, and a derivative of testosterone, 11-ketotestosterone (11-KT), is thought to be the main androgen in some teleost fish, such as fathead minnows (Ankley and Johnson 2004; Bentley 1998; Kime 1998). In both instances, testosterone is produced by Leydig cells in response to LH, and the androgens are responsible for male reproductive development, secondary sexual characteristics, and many courtship behaviours (Bentley 1998; Devlin and Nagahama 2002; Mayer *et al.* 1990).

The reproductive anatomy of the gonads is also conserved amongst vertebrates, but is more variable. The two fish species studied in this work, the fathead minnow (*Pimephales promelas*) and three-spined stickleback (*Gasterosteus aculeatus*) both have a slightly different testicular structure than that of mammals. In these teleosts, the testes are located along the dorsal body wall. Sperm ducts (or vas deferens) develop from the body wall and extend from both testes at the caudodorsal surface, leading to the genital pore located between the anal and urinary pores (Redding and Patino 2000). Unlike the mammals, no other ducts or accessory glands exist. The internal testicular structure of the fathead minnow and three-spined stickleback differ also from mammals; instead of seminiferous tubules, homologous lobules exist, separated from one another by a thin layer of connective tissue. The lobules are lined with Sertoli cells, forming a blood-testis barrier within which primary spermatogonia divide by mitosis to give rise to cysts: clusters of cells at the same stage of development. The cells in the cysts divide by mitosis and meiosis in the processes of spermatogenesis and spermiogenesis to form spermatozoa (Figure 1.4a). The dividing cells cause the cyst to enlarge and eventually rupture, releasing mature spermatozoa into the lumen of the lobule, which is connected to the sperm duct and exterior. The interstitial cells (Leydig cells), as well as fibroblasts, blood and lymph vessels, surround the lobules, in the same manner as in the mammalian testis (Leino *et al.* 2005; Wootton 1984).

The ovary of the fathead minnow and three-spined stickleback is also similar in structure to that of a mammalian ovary. The mammalian and teleost ovaries have homologous origins and are the site of egg production. They also both have ovarian ducts whose function is to allow passage of the eggs or foetus to the exterior, surrounded by smooth muscle to aid in this action. These ducts tend to have excretory functions and are oestrogen-dependent. However, the teleost ovarian duct originates as an extension of gonadal tissue, whereas the mammalian ducts and uterus derive from the Mullerian ducts. The ovaries are also similar between fish and mammals in that they are the sites where the germ cells reside, which divide by mitosis and then meosis to produce ova. In both cases, each ovum is surrounded by granulosa and theca cells, which collectively form an oestrogen-producing follicle. The ovum matures in the ovary and is released via ovulation by rupturing its follicle and bursting through the ovary wall into the ovarian cavity (Figure 1.4b). While the process of oogenesis is similar across all vertebrates, significant differences exist when considering the egg types, and sites of fertilisation and development (eg. oviparity versus viviparity).

In both the female fathead minnow and three-spined stickleback, the eggs are approximately 1 mm in diameter and are released directly into the environment for fertilisation and development (the species are oviparous) instead of being retained in a womb (viviparity). The ovaries are paired and asynchronous, meaning they have many oocytes at all stages of development. Female fathead minnows also have an enlarged urogenital papilla (a small conical tube located just anterior to the anal fin) for the deposition of eggs, which develops just before spawning. The maturation of oocytes in these fish involves a cortical alveolar stage, followed by vitellogenesis (the sequestering of the yolk precursor, vitellogenin, which is essential for embryo growth). Vitellogenesis is both oestrogen-dependent and unique to oviparous vertebrates, in which the yolk provides energy for the developing fry. Before they are ovulated, vitellogenic oocytes undergo maturation, a process in which the oocyte hydrates and the nucleus breaks down. When the fish are ready to spawn, the follicles rupture and the ova are released into the ovarian cavity and expelled by the female via the oviduct (Bentley 1998; Nagahama 1983; Wootton 1984).

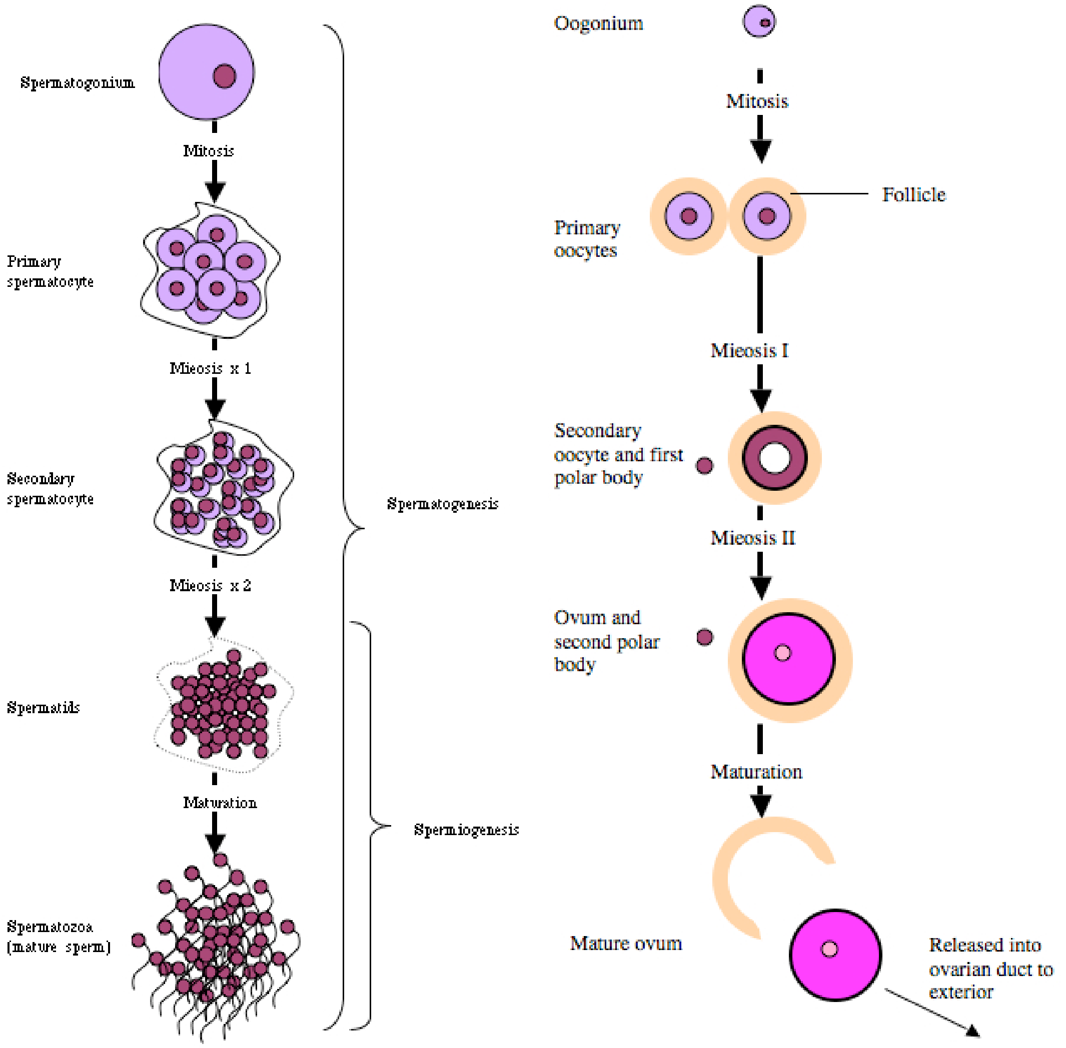


Figure 1.4. Depictions of (a) spermatogenesis within a cyst, and (b) oogenesis in the fathead minnow and three-spined stickleback.

In terms of ecotoxicology, the high degree of conservation of the endocrine systems between mammals and fish suggests that pharmaceuticals and chemicals able to elicit specific endocrine responses in humans and rats are likely to produce similar effects in fish. Thus, we would expect that since phthalates are anti-androgenic in mammals, they are highly likely to demonstrate similar activity in fish. Thus, the ubiquitous nature of phthalates in surface waters suggests they may be an important group of chemicals of concern to fish populations.

1.5.3. Considerations in choosing a specific phthalate ester for research

While several phthalates appear to have the ability to act as anti-androgens in mammals, in aquatic exposure studies, the behaviour of the chemical in water must be taken into account. While DEHP would seem the likeliest candidate to pose a threat to fish, because it is the most commonly encountered phthalate, its chemical properties suggest it might not pose as high a risk to aquatic organisms as some lower molecular weight phthalates. This is because DEHP saturates in water at a concentration of 3 μg/L, while BBP and DBP, both potent anti-androgens, are able to reach concentrations of approximately 10 mg/L. Thus, DBP was chosen as the phthalate of interest for this work, as it possesses several properties any chemical requires in order to be able to cause environmental endocrine disruption in fish: low general toxicity, moderate solubility in water, ability to bioconcentrate via the gills, evidence of endocrine disrupting activity, and documented presence in surface waters (Adams *et al*. 1995; Call *et al*. 1983; Foster 2006; Staples *et al*. 1997a; Staples *et al*. 2000; Staples *et al*. 1997b).

1.5.4. Existing research on the effects of phthalates on fish

Despite over ten years of investigation into the anti-androgenic effects of phthalates in mammals, initial studies of phthalates in fish were based on their potential estrogenic activity, following the finding of weak oestrogenicity of some phthalates *in* *vitro* (Harris *et al.* 1997; Jobling *et al.* 1995). However, it appears that phthalates do not have estrogenic effects *in vivo*, for example by inducing VTG and/or ovo-testis, or by altering fecundity (Harries *et al.* 2000). In general, much of the existing data should often be approached with a high degree of caution. This is because the majority of published studies have failed to conduct any form of analytical chemistry to support their conclusions, and hence exposure concentrations are unknown. Due to the difficulty encountered using phthalates in exposure studies (see Chapters 2 to 5), without well-conducted chemical analysis it is impossible to determine if fish are even exposed to phthalates, and if they are, at what concentrations. In the cases where actual concentrations are not measured, it is impossible to determine if any significant differences between "treated" and control groups are true responses to phthalate exposure, or whether the absence of a response is occurring despite the presence of a chemical. Analytical confirmation of exposure concentrations is therefore vital to any study of the potential effects of phthalate esters in aquatic biota. The results of studies lacking such data are discussed below, but they must be approached with the knowledge that any biological changes may not necessarily reflect concentration-responses to phthalates.

In terms of oestrogenic activity, examination of VTG production in both male and female fish exposed to DEHP and DBP has failed to produce any convincing results. Non-significant observations of VTG induction in males and reduction in females have been reported (Kim *et al.* 2002; Ortiz-Zarragoitia and Cajaraville 2005; Ortiz-Zarragoitia *et al.* 2006; Patyna and Cooper 2000). However, the majority of studies have failed to demonstrate any effects of phthalate exposure on VTG synthesis (Harries *et al.* 2000; Kim *et al.* 2002; Ortiz-Zarragoitia and Cajaraville 2005; Ortiz-Zarragoitia *et al.* 2006).

Small, non-significant increases in the incidence of ovo-testis in males have been observed following dietary DEHP exposure and aqueous DBP exposure (Norman *et al.* 2006; Patyna and Cooper 2000; Patyna *et al.* 2006). Generally, phthalate exposure does not appear to alter histological morphology of the gonads of fish (Metcalfe *et al.* 2001; Ortiz-Zarragoitia and Cajaraville 2005; Ortiz-Zarragoitia *et al.* 2006). In one experiment, however, histological analysis found significant changes in female gonads of Japanese medaka (*Oryzias latipes*) exposed to nominal water concentrations of 1, 10 and 50 μg DEHP/L. Tanks were dosed in a static renewal system 3 times a week from 1-2 days post-hatch (DPH) to 90 DPH. It should be noted that the authors did not sample the treatment water directly to ascertain exposure concentrations; instead they prepared a tank without fish and dosed it at the highest concentration (50 μg/L) for 3 days. They sampled this water and reported that the concentrations of DEHP were initially 88% of the nominal, but fell to approximately 57% after 72 hours. They did not test the lower concentrations of DEHP. It is well known that the saturation point for DEHP in water is 3 μg/L, based on several reports (Staples *et al.* 1997b), which is much lower than two of the nominal concentrations used. Regardless, histological examination revealed a significant reduction in the number of females with mature oocytes in the ovary, which was not concentration-dependent. While ovaries of 54% of control females contained mature oocytes (n=15), only 37, 0 and 22% of ovaries of females exposed to 1, 10 and 50 μg DEHP/L, respectively, contained mature oocytes (n=9, n=12, n=13). No histological effects on the testes of males were reported, but the authors also found significant reductions in the GSI of DEHP-treated females at the two higher concentrations (Kim *et al.* 2002). Other researchers that maintained BBP concentrations in a flow-through system at measured concentrations of 82 and 69 µg/L for 21 days found no effects on GSI of breeding pairs of fathead minnows (Harries *et al.* 2000).

Fecundity has been found to be significantly reduced or altered by early life-stage phthalate exposure in F_1 female Japanese medaka exposed to 776 µg DBP/kg/day via the diet from egg to maturation (Patyna and Cooper 2000). By contrast, it is less clear whether or not exposure during adulthood affects fecundity. There were no effects in females spawning with untreated males after 15 days of exposure to 100 and 500 µg DBP/L (Ortiz-Zarragoitia *et al.* 2006). This lack of an effect reported by Oritz-Zarragoita *et al.* (2006) should also be approached with caution. The females spawned with untreated males in clean water, and were only given 2-3 days to spawn, despite the fact that zebrafish generally require 5-10 days to spawn. Further, no analytical chemistry was conducted on this static system, with nominal concentrations exceeding the saturation point of DBP in water by 10- and 50-fold. Conversely, a more robust pair-breeding test that exposed adult fathead minnows to measured concentrations of 69 and 82 µg BBP/L found that while fecundity remained unchanged, the spawning frequency was significantly lower (Harries *et al.* 2000).

Very few researchers have specifically examined the potential of phthalates to act as anti-androgens in fish. However, exposure of fathead minnows to measured concentrations of BBP (69 and 82 µg/L) for 6 weeks found no effects on male fat-pad size or thickness, or nuptial tubercle number (Harries *et al.* 2000), all of which are considered androgen-dependent tissues. Another study aimed to examine changes in concentrations of sex steroids of carp, *Cyprinus carpio*, following 48h exposure to a range of concentrations of DEHP (5.5 to 20.5 mg/L). Pooled serum samples (n=1) of an unknown number of fish showed a strong concentration-related increase in testosterone and 11-KT. An inverse concentration-response relationship existed between phthalate concentration and plasma oestrogen concentrations in male and female pooled serum samples, which were significant at the higher concentrations (Han *et al.* 2009). However, no water chemistry was conducted and all of the concentrations stated are ~3-4 orders of magnitude higher than the saturation point of DEHP in water. This fact, combined with a lack of reporting on sample size, suggests the results should be approached with caution.

Changes to hormone concentrations in fish were also analysed in an *in vitro* study conducted on carp (*Cyprinus carpio*) testicular and ovarian microsomes (Thibaut and Porte 2004). Cultures of either testicular or ovarian microsomes were pre-incubated for 10 minutes with DBP or DEHP to analyse changes to steroidogenic and metabolic pathways. Testicular microsomes preincubated with 100 μM of DBP or DEHP and incubated with radiolabelled androstenedione, a testosterone precursor, did not exhibit reduced testosterone production, but concentrations of the testosterone derivative, 5α-androstene-3,17-dione, were significantly reduced by 45-65%. Only DBP had an effect on 5α-dihydrotestosterone production, reducing its concentration by 41%. This suggests that 5α-reductase, one of the enzymes responsible for production of 5α- androstene-3,17-dione and 5α-dihydrotestosterone, may be inhibited by these phthalates. By contrast, in female ovarian cultures, the production of 17 α-20α-dihydroxy-4-pregnen-3-

one and 17α,20β-dihydroxy-4-pregnen-3-one was increased after exposure to 1 mM of either DBP or DEHP, but only significantly with DBP (Thibaut and Porte 2004). While this study provides evidence for the potential of phthalates to inhibit androgens in fish, the *in vitro* use of androstenedione as a precursor for testosterone and its derivates may be somewhat misguided. Testosterone is produced from androstenedione via 17β-hydroxysteroid dehydrogenase, one of the few steroidogenic enzymes whose activity is not typically reduced by phthalate exposure in rats (Barlow and Foster 2003; Lehmann *et al.* 2004; Shultz *et al.* 2001). Further, the use of the parent compounds, DBP and DEHP, rather than their active metabolites, MBP and MEHP, is surprising; the parent compounds are generally inactive in *in vitro* assays since cell cultures lack the ability to metabolise the parent compounds to their active monoesters (Gray and Beamand 1984). Thus, a lack of reduced testosterone production in phthalate-exposed testicular microsomes may be likely due to the use of an inappropriate steroid precursor and inappropriate phthalates.

Overall, these papers demonstrate that while attempts have been made to assess the effects of phthalates on fish, few studies have utilized the extensive mammalian literature on the subject in order to target appropriate endpoints for the investigation of the anti-androgenic activity of phthalates in fish.

1.5.5. Experimental design and hypothesis

A solid experimental design is required to study phthalates, as they are difficult to use in aquatic exposure systems, and may have subtle and unexpected effects. The experiments discussed herein were designed using the mammalian studies as a guide in order to maximise the possibility of determining whether or not phthalates are able to act as anti-androgens in fish at concentrations present in the environment.

The general design of the experiments involved:

- aqueous exposure to fish (rather than dietary) in a flow-through system, to most accurately mimic environmental conditions downstream of point source inputs, such as sewage treatment plants.
- the use of environmentally-relevant concentrations of phthalates.
- the use of a commonly identified, well-known anti-androgenic phthalate ester, di-n-butyl phthalate, which has the potential to bioconcentrate in fish tissues.
- exposure to fish during critical windows of sexual development to mimic mammalian *in utero* exposure.
- the targeting of likely endpoints for anti-androgenic endocrine disruption in both female and male fish, such as hormone concentrations, gonadal histology, secondary sexual characteristics, steroidogenic gene expression, spawning and hatchability, and reproductive behaviours.

The aim of the work herein is to test the hypothesis that DBP acts as an anti-androgen in fish, disrupting gonadal histology and development, and altering plasma hormone concentrations, steroidogenic gene expression, and secondary sexual characteristics, ultimately affecting reproductive behaviour and success.

Chapter 2. Pilot Study: The effects of di-n-butyl phthalate on the fathead minnow in a two-generational study.

2.1 Introduction

Due to the general consensus that the effects of phthalates are most potent in developing mammals (Li *et al*. 2000; Li *et al*. 1998; Sjoberg *et al*. 1986; Wine *et al*. 1997), it seemed logical that if fish were susceptible to phthalate-induced endocrine disruption, the most sensitive period would be during their early development. This likelihood, combined with the relative lack of understanding of any clear mechanism of action of phthalates, suggested a two-generational study would provide the most useful design to elucidate the anti-androgenic effects of phthalates in fish.

2.1.1 The fathead minnow

The fathead minnow (*Pimephales promelas*) is a small freshwater cyprinid fish with a wide distribution across North America. It lives in both lakes and streams, and has a generalist diet consisting mainly of zooplankton, small plants and insects, and occasionally, small fish (Rook 1999). Its colouration ranges from olive to brown on the upper body and silver/white on the lower body, with a dark mid-lateral stripe (EPA 2002). Fathead minnows are gonochoristic and thus develop into either females or males.

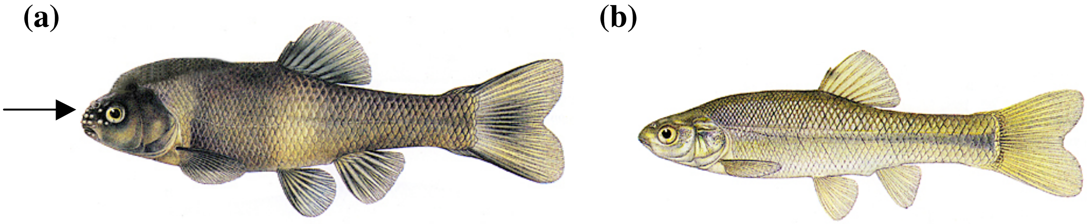


Figure 2.1. Mature fathead minnows (*Pimphales promelas*). (a) Male with a fat pad and nuptial tubercles (arrow), and (b) female. Illustrations by Joseph Tomelleri from Yonkos *et al.* (2000).

Adults typically reach maturity at approximately 120-150 dph, and reach a maximum fork length of approximately 48-70 mm (Ankley and Johnson 2004; Van Aerle 2004). They are also sexually dimorphic (Ankley and Johnson 2004). Females tend to be ovoid in shape and silver in colour. Males are distinctly larger than females, and have secondary sexual characteristics including a darker head and body, nuptial tubercles (small raised bumps on the snout), and a fatty pad on the top of their head (Figure 2.1) (EPA 2002). Males defend spawning territories against other males and maintain the fertilized eggs prior to hatch (Jensen *et al.* 2001). Both the secondary sexual characteristics and reproductive behaviour appear to be androgen-mediated (Miles-Richardson *et al*. 1999; Smith 1974).

2.1.2 Spawning

In the wild, spawning usually occurs from May to August. Spawning sites consist of nests maintained and protected by sexually mature males and are typically located underneath submerged stones and branches (Yonkos *et al.* 2000). In the laboratory, pairs can mate and spawn every 1-15 days, but do so more commonly every 3-4 days. Approximately 40-500 eggs can be deposited and fertilized by a pair in one spawning. Eggs typically take 4-5 days to hatch, depending on the temperature (Harries *et al.* 2000; Jensen *et al.* 2001).

2.1.3 Reproductive development

It is unclear exactly when the period of cellular differentiation occurs in the fathead minnow gonads. Histologically, female ovaries are considered to be identifiable as early as 10 dph, by the presence of centrally located meiotic germ cells surrounded by somatic cells, and a rudimentary ovarian cavity. By 25 dph, females are expected to have developed primary oocytes in their ovaries, and may have late vitellogenic oocytes as early as 74 dph, but more often by 88 dph (Panter *et al.* 2002).

Males are thought to develop more slowly, with all spermatogonia in the testes remaining quiescent until 60 dph, at which point the onset of mitotic divisions of the germ cells and the formation of testicular cysts can be observed. However, spermatozoa do not appear until between 88 to 150 dph (Panter *et al.* 2002; Van Aerle 2004). A previous study observed that a critical window for the feminization of the testicular duct in male fathead minnow had been established from 10-15 dph following exposure to 10 ng EE2/L (nominal concentration). Additionally, exposure to this concentration of EE2 from 24 hours post-fertilization to 20 dph or from 15-20 dph also appeared to result in reduced numbers of spermatozoa in the male testes (Van Aerle *et al.* 2002). Surprisingly, no incidences of intersex were found in that study, suggesting that the fish may not have been exposed during the critical period for spermatogenesis. Since the fish were not exposed to EE2 after 20 dph and it is known that spermatogenesis does not begin until 60 dph, it is possible that the critical window for the development of spermatogenesis may extend past 20 dph, perhaps to the time of sexual maturation of the male.

2.1.4 Fathead minnows as a model in endocrine disruption studies

The fathead minnow is a very good test species for assessing endocrine disruption for several reasons. It is a robust species, able to withstand variable conditions including muddy waters, low oxygen concentrations, and a wide range of pH (Rook 1999). Furthermore, the rapid spawning rate of the fathead minnow allows for testing the effects of chemicals on reproductive endpoints over short periods of time.

While other species of fish, namely Japanese medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*), are also considered to be very useful in aquatic toxicology testing, fathead minnows were specifically chosen for this pilot study for several reasons. First, fathead minnows, as opposed to medaka and zebrafish, are large enough to permit removal of enough blood for steroid analysis (although this is terminal). As phthalates are known to reduce testosterone production in mammals, the direct measurement of plasma hormone concentrations in fish should be a useful endpoint for anti-androgenic disruption. Secondly, secondary sexual characteristics in fathead minnow males are under androgenic control, and are potentially suitable endpoints to indicate reduced androgen levels caused by phthalate exposure (Ankley and Johnson 2004). Finally, the Institute for the Environment at Brunel University has considerable experience in using fathead minnows as a test species in the study of endocrine disruption. For all these reasons, this species was a logical choice for this initial experiment.

2.1.5 Experimental aim and design

The aim of this experiment was to determine if environmentally-relevant concentrations of di-*n*-butyl phthalate would induce anti-androgenic endocrine disruption in fathead minnows in a two-generational study: both in adult fathead minnows (F_0 generation) and in their offspring (F_1 generation). In the F_0 generation, we hypothesized that DBP exposure would cause reduced androgen production, resulting in changes to spawning behaviour and/or reproductive success. In both F_0 and F_1 generations, we aimed to examine whether or not the effects of DBP would cause reduced plasma androgen concentrations in males, alter the histology of the gonad, and interfere with the expression of the male secondary sexual characteristics.

Two-generation studies, while more complex, can be useful in elucidating reproductive effects in adults, as well as developmental effects in juveniles (Ankley and Johnson 2004). They also allow for potential exposure of the developing embryo to the chemical of interest through maternal transfer to the eggs. This is especially likely when studying chemicals that are able to bioaccumulate in the lipids, which are transferred to the egg yolk. In the case of phthalates, it appears that biomagnification and trophic transfer do not occur (Mackintosh *et al.* 2004). However, a low level of transference of phthalate esters to the egg lipids was measured in the embryos of wild-caught dogfish (Mackintosh *et al.* 2004).

Due to the lack of a discrete window of sensitivity for sexual development of males especially, the developing fathead minnows (F_1) were exposed for the entirety of the experiment, from egg to adulthood (150 dph).

2.2 Methods

2.2.1 F_0 Generation: Selection of fish

42 females and 42 males approximately 13 months of age (from laboratory stocks 0 and 9/10, hatched June 11th and 23rd, 2005, respectively) were used as the F_0 generation. Fish were randomly divided evenly among seven 30 L tanks (0.6m x 0.3m x 0.3m) to give a total of 6 females and 6 males in each tank. They were allowed to acclimatize over a period of 6 days prior to exposure to phthalates.

2.2.2 F_0 Generation: General conditions

The experimental system was set up first, by preparing stock solutions of DBP in dimethylformamide (DMF) (Fisher Scientific, UK), one per treatment tank. These were prepared in silanized brown glass bottles at volumes of 500 ml, designed to last approximately 2-3 weeks. With a peristaltic pump, stocks were pumped into glass mixing vessels at a rate of 0.01 ml/min. along with water from the header tank flowing at a rate of 500 ml/min. The contents of both the stock bottles and the mixing vessels were constantly stirred to ensure a homogenous distribution of DBP in the water. The DBP-dosed water then flowed, by gravity, from the mixing vessels directly into the tanks containing the fish (Figures 2.2 and 2.3). Final nominal concentrations of DBP in the tank water were 6, 12, 25, 50 and 100 μg DBP/L, with a final solvent concentration of 20 μl/L DMF solvent, as recommended by Hutchinson *et al.* (2006). DBP was allowed to flow through the fish tanks for 6 days, in order to stabilise concentrations before the fish were added.

Over the course of the experiment, the temperature was maintained at 25 ± 1°C. Each fish tank was aerated with two air stones. Oxygen concentration and water temperature were recorded daily, while the flow rates of both the water and stock solutions into the mixing vessels were measured twice weekly. Water samples were collected on four occasions during the experiment to measure the actual DBP concentrations. The lighting regime during the experiment consisted of 16 hours light and 8 hours dark. Adult fish were fed four times daily, twice with flaked tropical fish food (Tetra Inc.), and twice with live brine shrimp nauplii (*Artemia*).

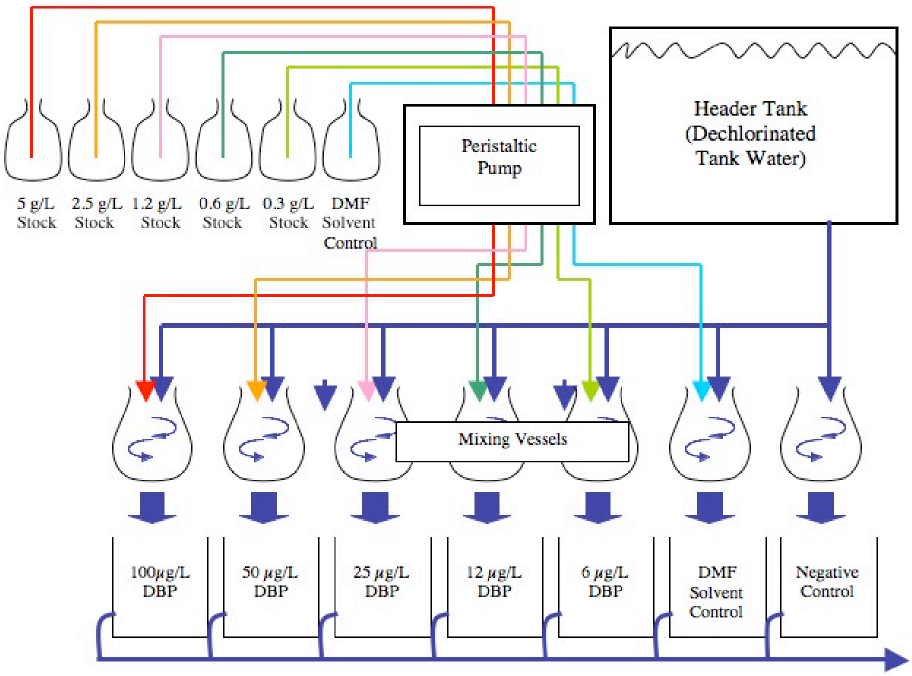


Figure 2.2. Depiction of the flow-through exposure system used to expose both the F_0 and F_1 generations of fathead minnows to various concentrations of DBP.

After the 6-day acclimation period, the fully mature fathead minnows were added to DBP-treated tanks. Each tank contained six nesting sites made of inverted semi-circular tiles sitting atop glass trays and covered by metal grids. When the fish spawned, most of the embryos would adhere to the underside of the tiles, but any that dropped off were collected in the trays underneath. This helped to prevent their consumption by the adults in the tank (Figure 2.4). The exposure of the F_0 generation was conducted over 21 days (July 31st to August 21st, 2006).

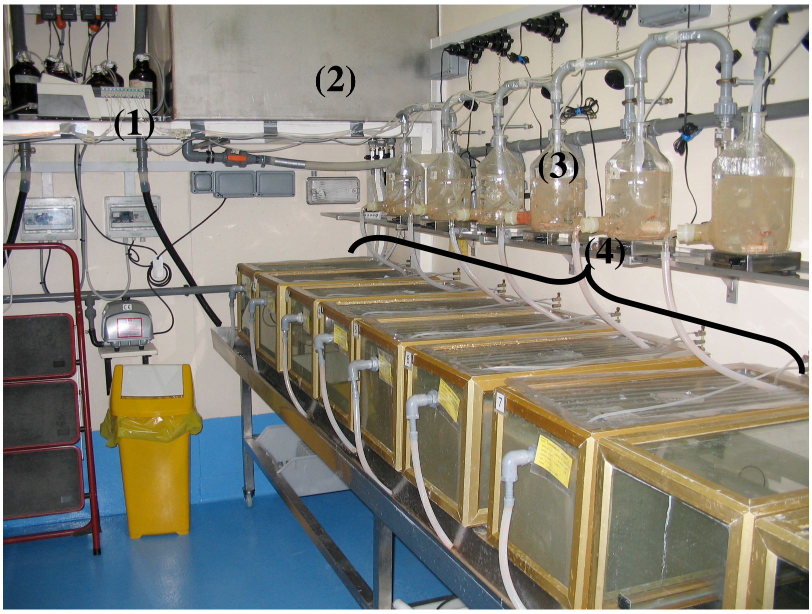


Figure 2.3. Photograph of the experimental system used to expose fathead minnows to DBP showing (1) the peristaltic pump and stock solutions, (2) the header tank holding the water (3) the mixing vessels, and the (4) the fish tanks.

2.2.3 F_0 Spawning

During the 21-day exposure period, the egg tiles and their trays were collected daily from the fish tanks, and placed into containers with fresh header tank water, in order to maintain embryo viability. Viable, non-viable eggs, and bound, and loose embryos were counted in water using a hand-held counter and a low-power dissecting microscope. Embryos were then discarded and clean trays and tiles were placed back in the tanks. Embryos produced in the final week of the F_0 experiment were kept to provide fish for the F_1 generation. These embryos were left attached to the tiles, and were placed in metal mesh baskets suspended in the water from the top of the F_0 fish tanks, in order to prevent adult predation while they developed.

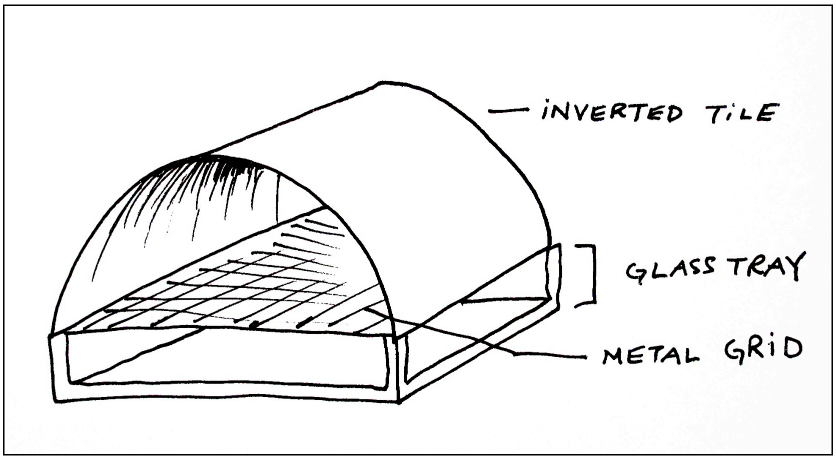


Figure 2.4. Diagram of spawning tile used in the F_0 (adult) exposure phase of the two-generation study of the effects of DBP in fathead minnows.

2.2.4 F_1 Generation: General conditions

Approximately two days after all the fry had hatched in the suspended baskets, the juvenile fathead minnows (F_1 generation) were moved to a separate set of fish tanks for continued exposure to DBP in the same manner as described for the F_0 generation. Lighting and temperature regimes were the same as previously described. The fish were exposed to the various DBP treatment concentrations until they reached 150 dph (January 16-18, 2007).

F_1 juveniles were fed with Liquifry No.1 (Interpet Ltd.) four times daily for the first 2-3 weeks post-hatch. They were then switched to a regimen of brine shrimp nauplii and Tetramin Powdered Baby Fish Food (Tetra Inc.) fed a total of four times a day, the latter of which was switched to Liquifry No.3 (Interpet Ltd.) when fish grew larger. At approximately 100 dph, when fish were almost mature, the F_1 fish were fed the same adult diet as F_0 fish (see Section 2.2.2).

2.2.5 Thinning of the F_1 generation

When the fish had reached 100 dph (November 29th, 2006), the F_1 generation fish were thinned to 50 fish per tank to control variation in density (Table 2.1). A table of 50 random numbers, generated by GraphPad Software Inc., was obtained out of the total number of fish in the tank. Fish were then caught in succession, and kept if the order in which they were caught corresponded to one of the 50 numbers. These 50 fish were then treated with DBP until they reached 150 dph. On the same day, a small number of fish (n=5), aged 100 dph, were taken from each tank for early histological analysis according to the methods described in Section 2.2.7. Fish were not sampled from the Solvent Control group at this time, due to the small number of fish in this tank (Table 2.1).

Table 2.1 Fish density in F_1 generation tanks before and after thinning, and the sample size of the fish collected at 100 dph for early histological sampling.

Treatment	Total Number Fish Initially Present	Number of Fish Sampled for Early Histology (100 dph)	Final Number of Fish in the Treatment Tanks at the End of the Experiment*
Water Control	85	8	44†
Solvent Control	40	0	38††
6 µg DBP/L	125	5	50
12 µg DBP/L	82	5	49
25 µg DBP/L	57	5	48
50 µg DBP/L	254	5	46†
100 µg DBP/L	55	5	48
Solvent Control 2	144	6	49†

*The target number of fish in each tank at the end of the experiment was 50. However, due to errors in the thinning and some fish loss, these numbers varied from their target value.
† 1 fish found dead in the tank prior to 150 dph.

Since the F_1 Solvent Control group had only 40 fish, we decided to supplement this group with an additional Solvent Control group. This was produced by assembling one tank containing 3 males and 3 females from the same stock used previously (stock 9/10), and allowing them to acclimatize for four days. A fish tank labelled, “Solvent Control—2” was assembled at the same time and DMF was pumped into the exposure tank at a concentration of 20 µl/L. The fish were transferred to this tank on September 19th, 2006. Embryos from this tank were collected and counted during the third week only, using the same methods as previously described (Section 2.2.3). These embryos were kept in suspended baskets and following the removal of adults, the fry were placed in the tank itself. Juveniles from the Solvent Control—2 tank were reared following the same methodology as the original F_1 generation. Again, at 100 dph, using a sample of 50 random numbers, the supplementary Solvent Control—2 fish were thinned from 144 to 49 fish at 100 dph (January 22nd, 2007) (Table 2.1). Of those removed, six fish were sampled for early histological analysis. Final sampling for the Solvent Control—2 tank was conducted when the fish had reached 150 dph (March 12th 2007).

2.2.6 Water chemistry

2.2.6.1 The principles of extraction and analysis of water samples

In order to properly study the effects of aqueous phthalate exposure in fish, it was necessary to conduct chemical analysis of the tank water to confirm that the fish were exposed to DBP at the intended concentrations, and to ensure the control groups were not exposed to this chemical.

Water chemistry is often a two-step process involving, first, the extraction of the chemical from the water sample, and second, analysis of the chemical, involving both identification and quantification. A popular method for extracting low concentrations of lipophilic substances from water is reverse-phase solid-phase extraction (SPE). In this process, water samples pass through a SPE cartridge containing a highly lipophilic substance, such as octadecylsylane (C18), which binds any non-polar chemicals to its surface. The water therefore is "stripped" of any non-polar chemical it contains (in this case, phthalates), which is collected in the SPE cartridge. The chemical can be washed from the C18 cartridge by eluting it with an organic solvent such as methanol, acetone, or in this case, hexane. Once the chemical is collected in the solvent, the solvent is evaporated off, leaving (theoretically) 100% of the chemical from the water sample. This is then reconstituted with a carrier solvent to a known volume, and injected into an analyser, such as a gas chromatographer-mass spectrometer (GCMS), which identifies and quantifies the chemical (Figure 2.5).

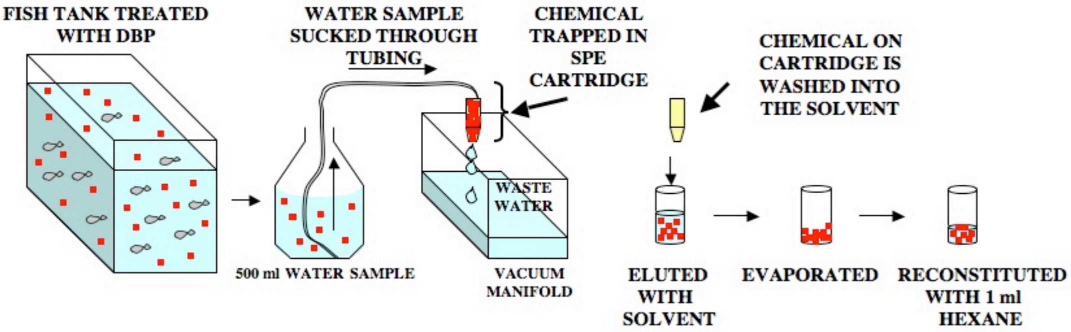


Figure 2.5. Depiction of the reverse-phase solid-phase extraction method. The DBP (depicted as red dots) is extracted from the water sampled from a fish tank onto a SPE cartridge, and then reconstituted in hexane so that it can be injected on a GCMS.

GCMS involves two steps: a separation phase (gas chromatography or GC) and an analytical phase (mass spectrometry or MS). The gas chromatographer separates all of the different chemicals in a sample from one another based on their physical and/or chemical properties. The chemicals are vaporized in the machine and carried by a mobile phase, in this case helium. They are then run through a stationary phase, which, in our case, was a column packed with glass fibres. The larger particles take longer to move through the stationary phase, while smaller particles will move more quickly and elute faster (Figure 2.6).

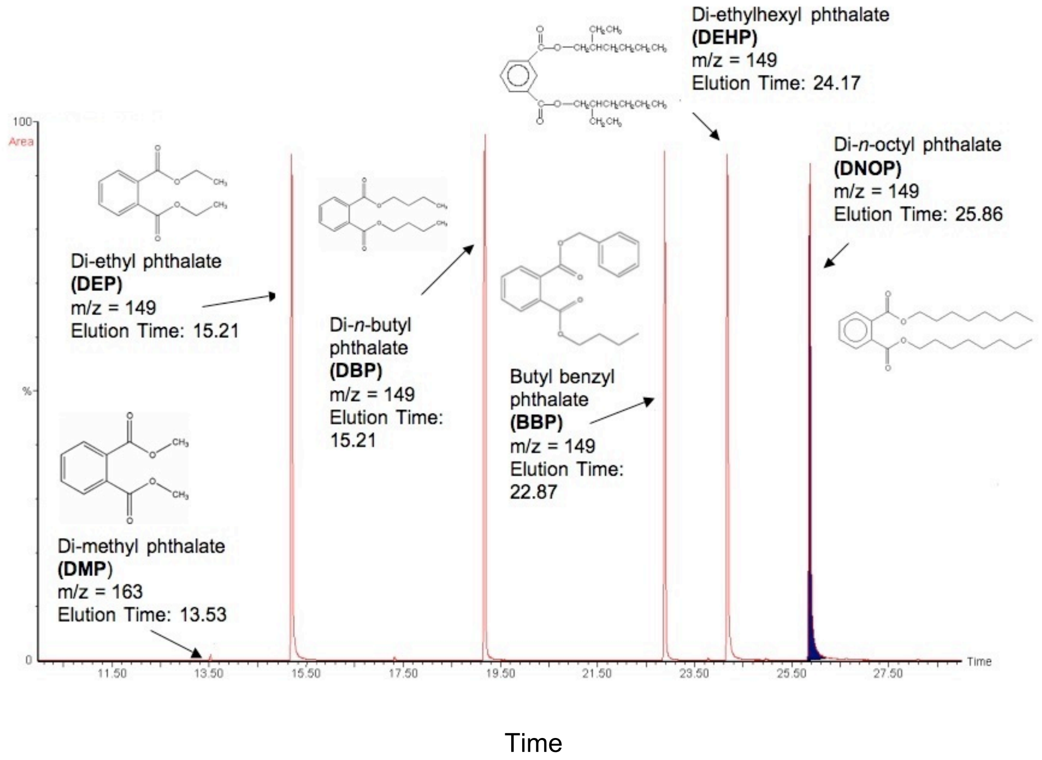


Figure 2.6. Chromatogram of the Phthalate Standard Mixture run on the GCMS and screened at 149 *m/z*. This mixture contains 40 mg/L of each of the phthalates DMP, DEP, DBP, BBP, DEHP, and DNOP. (The DMP peak is very small because its major fraction is 163 *m/z*, not 149 *m/z*). The peaks of the phthalates are separated by their elution time on the GC, and their peaks are quantified by the MS. The unique molecular structure of each phthalate ester is shown above.

Once the sample has travelled through the GC, the separated chemicals reach the mass spectrometer. A mass spectrometer is a machine which ionizes and fragments different molecules by bombarding them with electrons. The MS sends these charged fragments through a magnetic field toward a detector which detects the charge or current created by each ionized particle, quantifying them as mass-to-charge ratios (*m/z*). Each fragment has a specific *m/z* and each chemical usually has several fragments, which collectively form a unique combination of *m/z* ratios, like a fingerprint. Similar chemicals such as phthalates tend to have similar fingerprints, and thus the elution time of the GC is very important in identifying each phthalate individually (Figure 2.6). The MS also quantifies the amount of each chemical detected in a sample as a peak. Based on the peak area, the mass spectrometer determines the concentration of each chemical in the sample by comparing it to the peak areas of the standards run at the beginning and end of each GCMS run (Figure 2.7).

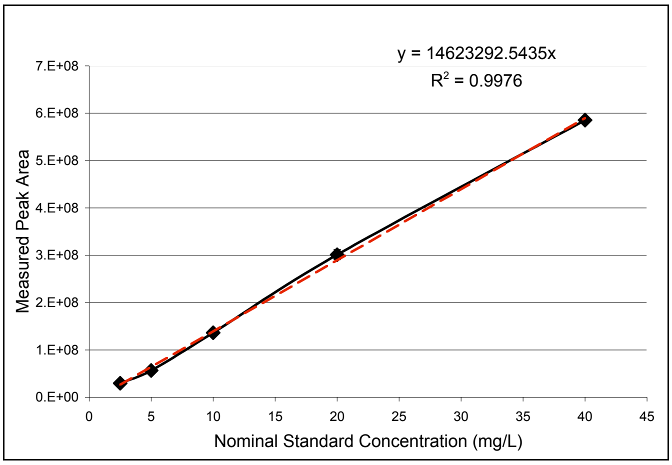


Figure 2.7. Standard curve of a set of DBP standards measured by GCMS. Peak area is plotted against the nominal concentrations of the standards, and calculated by linear regression (red dotted line).

2.2.6.2 Water chemistry protocol

The water samples for analytical chemistry were originally sent to a collaborator for analysis. Due to complications, the first set of samples were reported to have no measurable concentrations of DBP. A second set was sent to confirm these results, but they were never analyzed. Consequently, all future water samples were measured by the author, and were only collected and analyzed on four occasions during the six-month experimental period (on Days 0, 136, 156, and 171 of the 171-day experiment) (Figure 2.8).

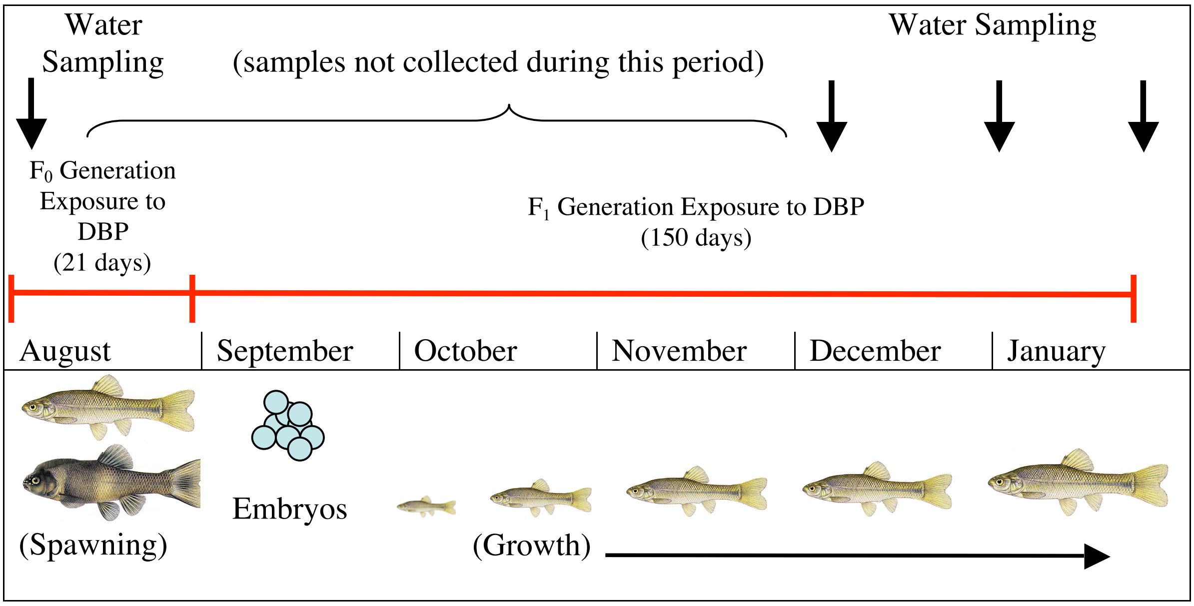


Figure 2.8. Depiction of the general time line for the exposure of both the F_0 and F_1 generations of fathead minnows to various concentrations of DBP. The relative times when water samples were collected are indicated above by arrows.

Water sampling involved the collection of 500 ml water from each fish tank with clean, rinsed volumetric flasks, which were then decanted into silanized 1L brown glass bottles. Prior to extraction, 5 ml of HPLC-grade methanol (Fisher Scientific Ltd.) was added to each water sample. The Sep-Pak Plus C18 extraction cartridges (Waters Ltd., UK), pre-conditioned with 5 ml methanol and 10 ml deionized water (Milli-Q), were placed on a vacuum Manifold. Care was taken to ensure the cartridges did not dry out during any of the extraction process, since this can reduce the efficiency of the cartridge to extract chemicals from water.

The water samples were drawn through methanol-washed Teflon-lined tubes, and through the cartridges at an approximate rate of 3 ml/min. This took from 20 minutes to 3 hours, depending on the amount of suspended particulate matter in the sample. Once all of the water sample had passed through the SPE cartridges, they were dried with air, and placed in the freezer at -20°C to await elution. Prior to elution, SPEs were left to thaw at room temperature for approximately 30 minutes, and were then eluted with 7 ml HPLC-grade hexane (Rathburn Co.) into clean glass vials. The hexane was evaporated off under a stream of nitrogen at room temperature. Once dry, the sample was reconstituted with 1 ml hexane, sealed with parafilm, and stored at 4°C. These samples were measured in duplicate on the Clarus 500 GCMS (Perkin Elmer Ltd.).

The GCMS was run by the author on November 26th 2007. A volume of 1.0 μl of each sample was injected in the split mode (1:2 ratio) at 250°C into a 30 m x 0.25 mm x 0.25 μm BPX5 column (SGE Analytical Science Ltd.). The helium carrier gas flowed at a rate of 1 ml/min. The following temperature programme was used: 50°C for 1 min. increased at 10°C/min to 285 °C, and maintained at 285°C for 10 min. The quadrupole mass spectrometer operated in electron impact ionization mode at 70 eV. The transfer line and the injector were set up at 250°C and the source was 180°C. Measurements on the GCMS were performed in the single-ion monitoring (SIM) mode: 163 *m/z* for the phthalate, DMP, and 149*m/z* for DEP, DBP, BBP, DEHP, and DNOP. Peak areas of the samples were quantified by comparing them to the serially diluted standards (Phthalate Esters Mix, 2000 μg/ml in hexane, Sigma-Aldrich Inc.). These had been diluted in hexane to concentrations of 2.5, 5, 10, 20, 40 mg/L (kept at 4°C) and were injected directly onto the GCMS. These standards were run at the beginning and end of each run, with one standard repeated every 5 samples.

2.2.7 Sampling of F_0 and F_1 fish

Fish were collected and anesthetized with ethyl 3-aminobenzoate methanesulfonate salt (MS-222, Sigma-Aldrich Co.) until they were found not to exhibit uprighting behaviour. Each fish was given a reference number. Blood samples were collected from the severed caudal peduncle in heparinized capillary tubes containing a small droplet of aprotinin (Fisher Scientific UK Ltd.). Heparin prevents coagulation of the blood, and aprotinin prevents protein degradation in the plasma by blocking protease action. The blood samples were kept on ice until further processing. Each fish was then weighed and fork length was measured.

For the F_0 fish, sex was recorded along with fat pad weight, and nuptial tubercle number and prominence in males according to Smith (1978). Prominence was determined by visual examination based on protrusion and sharpness. The fat pad was removed by gently cutting it from the top of the head using a scalpel, taking care not to cut off any of the underlying muscle tissue. Gonads were removed by dissection and the gonad weight was measured. One gonad was fixed in ~5 ml Bouin's solution (Sigma Aldrich Inc.) for histological analysis, and the other was snap frozen in liquid nitrogen for molecular analysis (stored at -80°C).

For the F_1 fish, sex was recorded (where possible), and blood samples were collected using the method described above. Fat pad weight and tubercle numbers were not measured in F_1 males, due to the absence of such features in these young fish. Sex was determined by macroscopic examination of the gonads within the body cavity and the entire fish was placed in a labelled vial containing ~25 ml Bouin's solution.

Within ~3 hours of collection, the blood samples kept on ice were centrifuged at 12,500 g for 5 minutes. Then 20 μl of plasma from F_0 generation fish, and 5, 10, 15 or 20 μl of plasma from F_1 generation fish (depending on the available volume) was aliquoted to a new tube. Ethyl acetate (Sigma-Aldrich Inc.) was added to the samples at a 1:25 ratio (volume of plasma to ethyl acetate) and stored at -20°C.

2.2.8 Radioimmunoassay of steroid hormone concentrations in blood plasma

2.2.8.1 Principle of radioimmunoassays

Immunoassays determine the concentration of a particular substance in a sample using the reaction between an antibody to its specific antigen, in this case the antigen being our hormone of interest, 11-KT. Radioimmunoassays use radiolabelled antigens (for example, tritiated 11-KT) to compete with unlabelled antigens (the 11-KT from the blood samples) for a limited amount of binding sites (antibodies) over 24 hours. After separation of the unbound and antibody-bound fractions, the bound fraction is then analyzed for radioactivity and compared with a standard curve (Figures 2.9 and 2.10). Thus, the more bound radiolabelled steroid in the sample, the less unlabelled steroid is present and vice versa. In other words, the amount of radioactivity is inversely proportional to the amount of target steroid in the sample, which is back-calculated using a standard curve (Figure 2.10).

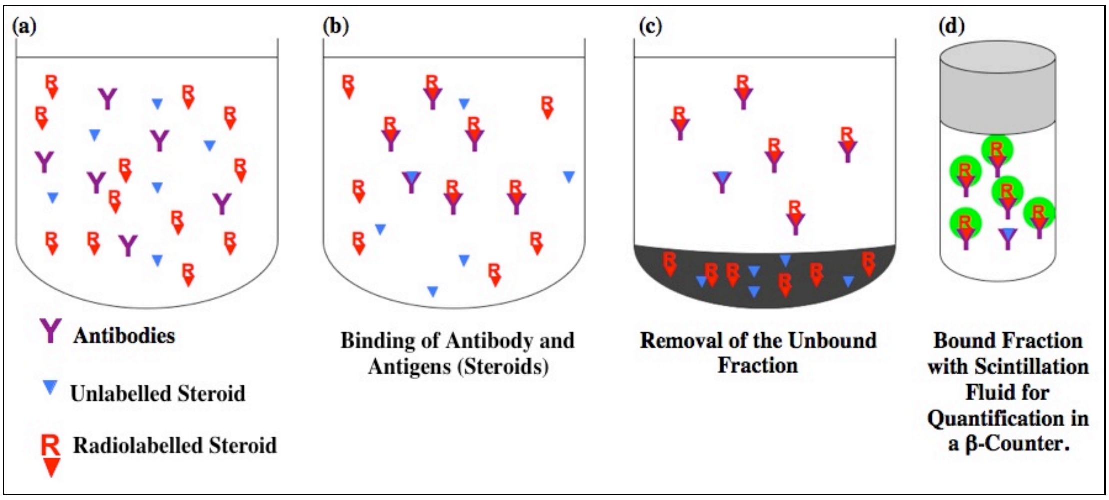


Figure 2.9. Depiction of the principle of radioimmunoassay. (a & b) Over time the antigens bind to the limited amount of antibody in proportion to the amounts of each that are present. (c) The unbound steroids are removed with activated charcoal, and (d) the sample is placed into a vial with scintillation fluid which fluoresces when β-particles are emitted by the radiolabelled steroid; the fluorescence is quantified by a β-counter.

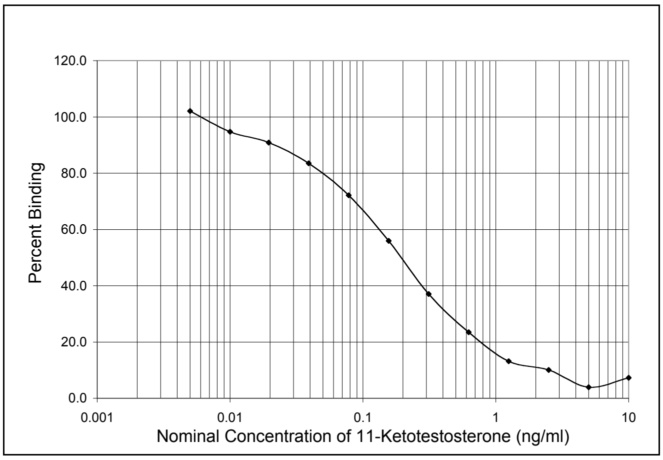


Figure 2.10. A standard curve for the 11-KT radioimmunoassay. The nominal concentrations of the diluted 11-KT standards are plotted against the Percent Binding, which is inversely proportional to the amount of radioactivity. The slope of the linear part of the curve (usually between ~20-80%) is then used to calculate the actual concentrations of 11-KT in each sample.

2.2.8.2 Materials and components

- Steroid Assay Buffer (SAB):
  - 1 litre dH_20 made to a concentration of 0.05 M phosphate buffered saline (10 tablets, Dubelco Ltd.)
  - 1 g gelatine (Type A from porcine skin; Sigma-Aldrich Inc.)
  - 1 g sodium azide (Sigma-Aldrich Inc.)
  - Using a heated stirrer, gelatine and PBS tablets were dissolved in the water. Once cooled sodium azide was added and mixed well. The buffer was then stored at 4°C.

- Antibody: Dilutions for each steroid of interest were prepared in SAB as follows:
  - 11-Ketotestosterone antibody was diluted 1:50,000
  - Testosterone antibody was diluted 1:50,000

    - 17β-Oestradiol antibody was diluted 1: 40,000
- Radiolabel:
    - Tritiated steroids (11-KT, testosterone, and oestradiol) were prepared by, and purchased from Amersham International.
    - Aliquots were prepared in SAB at radioactivities of approximately 6000 dpm per 100 μl for use in the assays. All were stored at -20°C.
- Standard:
    - Solutions of 11-KT, testosterone and E2 were prepared in aliquots of 250 μl at concentrations of 10 ng/L and stored at –20°C.
- Activated Charcoal Solution: prepared at 0.5% activated charcoal (Sigma-Aldrich Ltd.)with 0.05% dextran (Sigma-Aldrich Ltd.) at least 15 minutes prior to use.
- Liquiscint Scintillation Fluid (National Diagnostics Ltd.)

2.2.8.3 Radioimmunoassay method: Day 1

Radiolabelled 11-KT, antibody and 11-KT standard were removed from the freezer and defrosted on ice. Tubes were prepared in duplicate in batches of no more than 50 tubes (25 samples). An entire assay generally consisted of no more than 4 batches.

Each plasma sample containing ethyl acetate was centrifuged at 12,500 g for 5 minutes. Then 100 μl of the ethyl acetate that had been added to each plasma sample (see Section 2.2.7) was dispensed into each of two duplicate tubes. The ethyl acetate was then evaporated using a concentrator (miVac, Genevac Ltd.). Once evaporated from the tubes, 100 μl SAB was added to each tube to resuspend the hormone. SAB was also added to all other tubes according to Table 2.2 and the standard curve and quality control tubes were then centrifuged at 500 g for 5 seconds to ensure all liquid was at the bottom.

Table 2.2. Volumes of various components added to the different tubes in the steroid radioimmunoassay.

Tube	Volume (ul)				
	SAB	Antibody	Label	Standard	Sample
Total Counts	200		100		
Non-Specific Binding	200		100		
Maximum Binding	100	100	100		
Standards	100 (not first tube)	100	100	100 (1st and 2nd tubes only)	
Samples		100	100		100 (in SAB)

The standard curve samples were prepared by serially diluting the steroid standard (in this case, 11-KT) 11 times to yield nominal concentrations of 10, 5, 2.5, 1.25, 0.625, 0.313, 0.156, 0.078, 0.04, 0.02, 0.01, and 0.005 ng/ml.

One hundred microlitres of the appropriate steroid radiolabel was added to all of the tubes, which were vortexed. Then 100 μl of antibody was added to all of the tubes (except total counts and non-specific binding). The tubes were then vortexed and centrifuged briefly to ensure all components were at the bottom of the tube. They were covered with a clean paper towel, and incubated overnight at 4°C.

2.2.8.4 Radioimmunoassay method: Day 2

After 24 hours incubation, the tubes were removed from the fridge and placed on ice; 500 μl SAB was added to the Total Counts Tubes. Then, 500 μl activated charcoal solution (0.5%) was added as quickly as possible to each tube of one batch. All tubes were vortexed, and incubated on ice for 10 minutes (counted from the addition of the charcoal solution to the first tube). At exactly 10 minutes, the tubes were spun in a pre-cooled centrifuge (4°C) for 15 min. at 2,500 g. After centrifugation the supernatant was carefully drawn off from the tube, and decanted into a clean scintillation vial. Four millilitres of scintillation fluid was added to each vial, and the vials were shaken vigorously.

2.2.8.5 Measurement and analysis

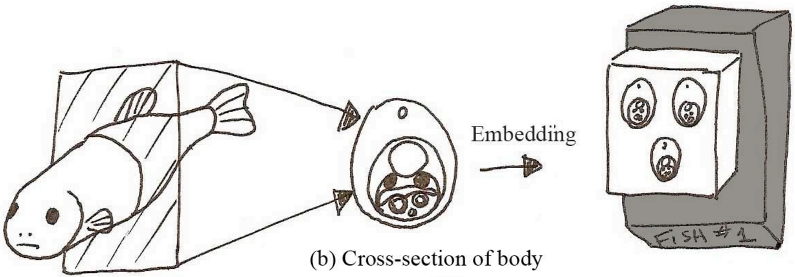
Radioactivity in the samples was counted for 5 minutes using a Liquid Scintillation Counter (Packard Bioscience Co.). The amount of radiolabel which binds to the antibody in the standards and sample tubes is expressed as a percent of the normalized Maximum Binding for each batch. This was determined using the following calculation:

$$\%\ \text{Binding} = \frac{(\text{mean sample decays per minute} - \text{mean Non-Specific Binding})}{(\text{mean Maximum Binding} - \text{mean Non-Specific Binding})} \times 100$$

2.2.9 Histological methods

2.2.9.1 Principle

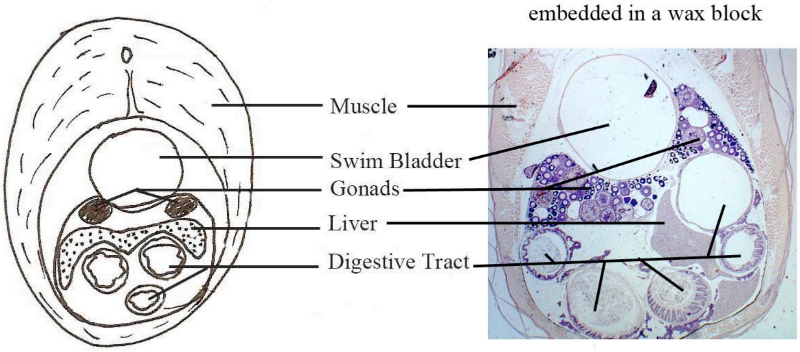
Histology is the study of the microscopic structure of cells. Preparation of tissues for histological examination is a process in which tissues are preserved in fixative to maintain the cell structure before being dehydrated and embedded in paraffin wax. The tissues are then sliced very thinly, and these sections are placed on glass slides and stained, so that they can be examined under a light microscope. In this case, the tissues were stained first with haematoxylin, which stains nuclear material dark blue or purple, and then with eosin, which counter-stains collagen, muscle, cytoplasm, and erythrocytes a pink colour (Figure 2.11).



(a) Whole fathead minnow

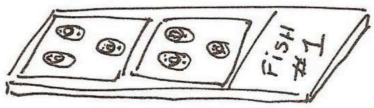
(b) Cross-section of body cavity

(c) Three sections of the fathead minnow embedded in a wax block



(d) Enlarged drawing of a whole-body cross-section

(e) Photograph of a female fathead minnow in cross-section



(f) Two sections from the wax block mounted on a glass slide.

Figure 2.11. Drawing of the histological processing of a fathead minnow (a & b) showing how the tissue is cut, (c) how the tissue is embedded on a wax block, (d & e) the identity of the various tissues in cross-section, both drawn and in a histology sample examined under a light microscope, and (f) how the microtomed wax section appears on a glass slide before it is stained.

2.2.9.2 Sample processing

The F_0 and F_1 histology samples were stored in Bouin's solution, which was replaced by 70% industrial methylated spirits (IMS) after 24 hours, and again after 48 hours. The gonads of the F_0 generation fish were sliced into five ~0.5 cm transverse sections with a microtome blade, and the 1st, 3rd and 5th sections were kept for embedding. Whole bodies of F_1 fish were sliced into five transverse sections

~0.5 cm in width from behind the operculum to the urogenital opening. Again the 1st, 3rd and 5th sections were placed into a plastic tissue cassette submerged in 70% IMS for histological processing (Figure 2.12).

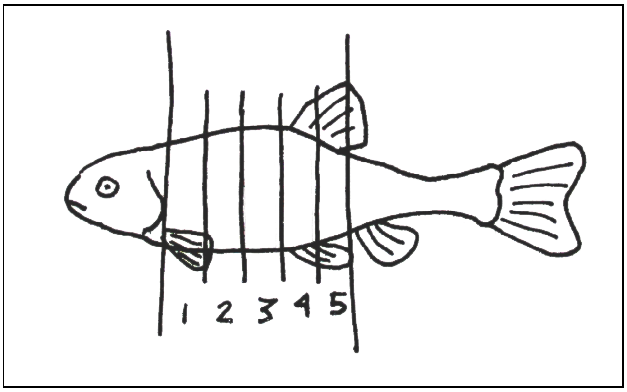


Figure 2.12. Diagram depicting the sections 1, 3, and 5 that were kept for histological analysis of the F_1 fathead minnows.

Tissues were processed according to the protocol in Table 2.3, using the automatic tissue processor (TP 1020, Leica Inc.), and subsequently embedded into wax blocks. Tissues were sectioned to 3 μm thickness using the RM 2235 microtome (Leica Inc.), and placed onto microscope slides coated with Histobond (RA Lamb, UK). They were dried for ~48 h before staining with Eosin and Haematoxylin according to the protocol in Table 2.4. Finally, the stained sections were mounted using Histomount (National Diagnostics, USA) and covered with coverslips.

Table 2.3. Summary of the tissue processing method of the Leica Tissue Processor, indicating the various solutions used and the time of each step.

Order	Solution	Time (hours)
1	70% IMS	3
2	90% IMS	2.5
3	95% IMS	1.5
4	100 % IMS	1.5
5	100 % IMS	1.5
6	100 % IMS	1.5
7	100 % IMS	1.5
8	Histoclear (National Diagnostics, USA)	1.5
9	Histoclear	1.5
10	Histoclear	1.5
11	Paraffin Wax (RA Lamb Inc.)	1.25
12	Paraffin Wax	1.25

Table 2.4. The staining protocol for histological slides of whole-body cross sections of fish.

Solution	**Time**	**Function**
Histoclear	15 minutes	Dissolves wax
100% IMS	2 minutes	Rehydration
90% IMS	2 minutes	
70% IMS	2 minutes	
Water	2 minutes	
Haematoxylin (VWR Inc.)	15 minutes	Stains nuclear material
Water	15 minutes	Wash
Acid Alcohol (HCl/70% IMS (1:99))	5-30 seconds	Resolves stain
Water	20 seconds	Wash
Saturated Solution of Li_2CO_3 (Sigma-Aldrich, UK)	20 seconds	Raises pH and removes any Bouin's residue
Eosin (RA Lamb, UK)	5-20 seconds	Counter-stains
Water	5 minutes	Wash
70% IMS	2 minutes	Tissue dehydration
90% IMS	2 minutes	
100% IMS	5 minutes	
Histoclear	5 minutes	Helps mount coverslip

The slides were examined under an Olympus BX51 compound light microscope and photographs were taken using a digital camera and the Q-Capture

Pro v. 5.1.1.14 program (Media Cybernetics Inc.). Examination of all histological samples was conducted blindly, without any knowledge of the sample identity or its treatment. All gonads were examined to identify the developmental stage, for the presence of cells in all stages of gametogenesis, and for any evidence of intersex (spermatogenic cells in the ovaries, or oogenic cells in the testes). Staging of the gonads and cell identification was conducted according to EPA (2009).

The males were specifically analysed for changes in the appearance of the interstitium, and for any signs of altered sperm duct development, or an ovarian-like cavity. The female ovaries were examined for the presence of oocyte atresia, a process in which oocytes (at any point in development) degrade and are resorbed. Atretic oocytes are characterized by clumping and perforation of the chorion, fragmentation of the nucleus, and disorganization of the ooplasm, among other factors (EPA 2009). The ovarian cavity of all females was also analysed for any signs of abnormal development, such as a lack of columnar appearance to the cells (Leino *et al.* 2005; Van Aerle 2004).

2.2.10 Statistical analysis

Statistical analysis was conducted using SigmaStat and SPSS statistical programs (version 3.5, Systat Software Inc., Germany, and IBM Inc., USA, respectively). Since the fish in the F_1 Solvent Control—1 and 2 groups had been exposed to the same conditions, the data from these groups were often pooled together and noted as “Solvent Controls”. This was only conducted if both Solvent Control groups were not statistically significantly different from one another, tested either by t-test or Mann-Whitney Rank Sum Test depending on whether or not they were normally distributed. The DBP-exposed fish were not compared to the Negative Control group.

One-way analysis of variance (ANOVA) was performed on normally distributed data or by Kruskall-Wallis ANOVA on Ranks where necessary. Significant results from one-way ANOVAs and ANOVAs on Ranks were followed by Holm-Sidak pairwise multiple comparison and Dunn's Test, respectively, to determine which groups were significantly different from one another. Analysis of Covariance (ANCOVA) was also used to analyse the effects of DBP on length and weight. Jonckheere-Terpstra Tests were used to analyse trends in F_0 male plasma 11-KT. Kaplan-Meier Survival Analysis (Log-Rank) was used to analyse the timing of spawning in the F_0 generation fathead minnows. Chi-square was used to determine differences in F_1 sex ratios. A significance level was set at $\alpha=0.05$ for all tests conducted.

2.3 Results

2.3.1 General conditions

Throughout the course of the entire experiment (both F_0 and F_1 generations), the oxygen and temperature were maintained at 7.45 ± 0.98 mg/L and 26.3 ± 15.6°C (mean ± SD), respectively, and the flow rate of the stock solutions into the mixing vessels remained constant at 0.01 ± 0.0006 ml/min (mean ± SD).

2.3.2 Measurement of actual phthalate concentrations

The GCMS was run on November 26, 2007, with replicates of one standard and one repeated sample every 5 runs. The results suggested a strong linear regression in the standard curve between measured and nominal concentrations (R^2 >0.99), with within-sample coefficients of variation ranging from 9 to 25% in the standards.

Measured concentrations of DBP in the DBP-treated fish tanks were highly variable between sample dates. Initially, on Day 0, the measured concentrations were close to nominal concentrations, however by December 2007 (Day 136), they were half of what was expected. On January 3rd, 2008 (Day 156), the concentrations were, at maximum, 10% of their nominal values, but interestingly, the concentrations of DBP did appear to have increased in the tanks on January 18th, 2008 (Day 171), when all of the fish, (except those from the Solvent Control—2 group), had been removed and sampled (Figure 2.13).

No contamination by the phthalates DEP, DMP, BBP, or DnOP was observed in any of the water samples collected during the experiment. However, DEHP was measured with a very high incidence (95%) in samples, but with trace concentrations of only 0.36 ± 0.16 μg DEHP/L (mean ± SD). In terms of DBP, 100% of the Negative and Solvent Controls had very low concentrations of the phthalate of interest (0.03-0.33 μg DBP/L) for the duration of the exposure. The mean concentration of DBP measured in the pooled Solvent Controls was 0.8 ± 0.8 μg DBP/L (SD). It was unclear, if the DEHP and DBP contamination were in the water samples or resulted from the sample processing and extraction instead.

Overall, the average DBP concentrations in each of the fish tanks are summarized in Table 2.5. Despite the disparity between measured and nominal concentrations in this experiment, the nominal concentrations will be used to identify the different treatment groups throughout the rest of this chapter. The apparent discrepancy between the nominal and measured concentrations is discussed later.

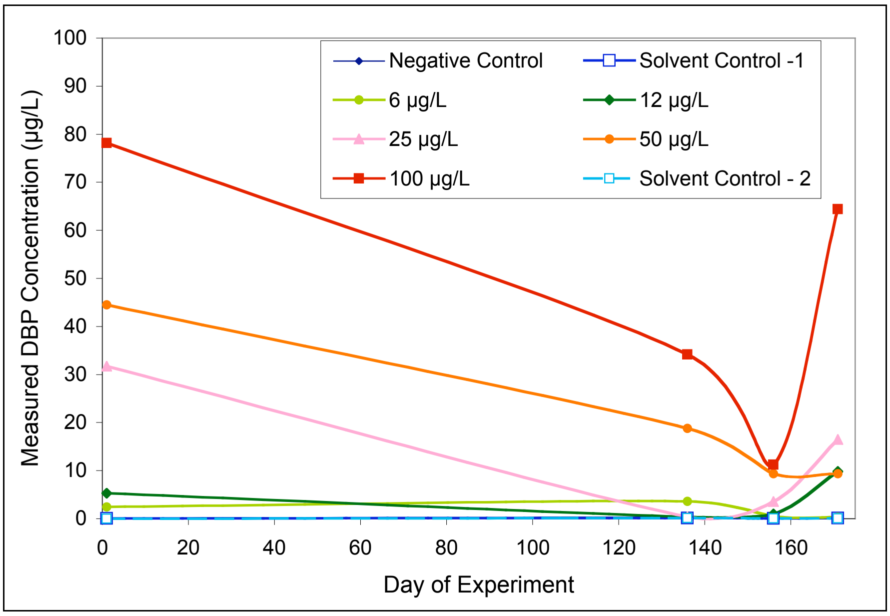


Figure 2.13. Concentrations of DBP in the water collected from the fish tanks during the exposure of F_0 and F_1 generations of fathead minnows to various concentrations of DBP.

Table 2.5. Measured concentrations of DBP (mean ± SD) in fish tanks over the course of the experiment exposing F_0 and F_1 generations of fathead minnows.

Treatment	DBP Concentration (Mean ± SD)
Negative Control	0.014 ± 0.13
Solvent Control—1	0.09 ± 0.04
Solvent Control—2	0.07 ± 0.04
6 μg DBP/L	1.72 ± 1.61
12 μg DBP/L	4.12 ± 4.39
25 μg DBP/L	13.02 ± 14.28
50 μg DBP/L	20.50 ± 16.62
100 μg DBP/L	46.99 ± 30.10

2.3.3 F_0 Generation fathead minnows

During the experiment, mortality occurred in two tanks: one male in the 12 µg DBP/L tank, and another male in the 50 µg/L tank. This did not appear to be related to DBP-exposure. All other F_0 fish appeared healthy throughout the experiment.

There appeared to be no effect of DBP treatment on the gonadosomatic index in either the males or females (P>0.05, df. 39 (male), df. 41 (female), ANOVA) (Figure 2.14). Similarly, lengths and weights of the fish did not appear to be affected by DBP treatment in either sex (P>0.05, df. 39 (male), df. 41 (female), ANCOVA) (Figure 2.15).

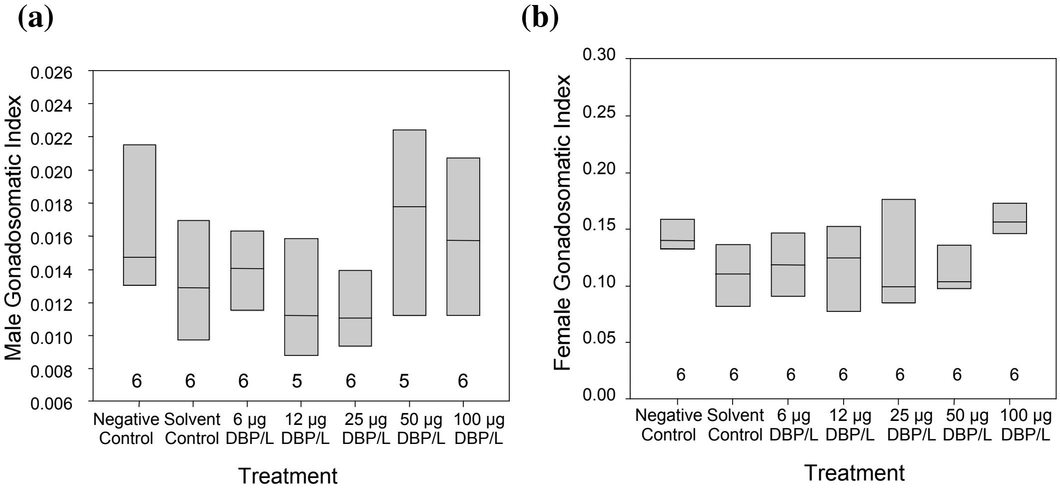


Figure 2.14. Box plots of the gonadosomatic indices of the F_0 generation (a) male, and (b) female fathead minnows after exposure to various concentrations of DBP for 21 days. (Boxes indicate the 25th and 75th percentiles about the median, and sample numbers are shown below each box).

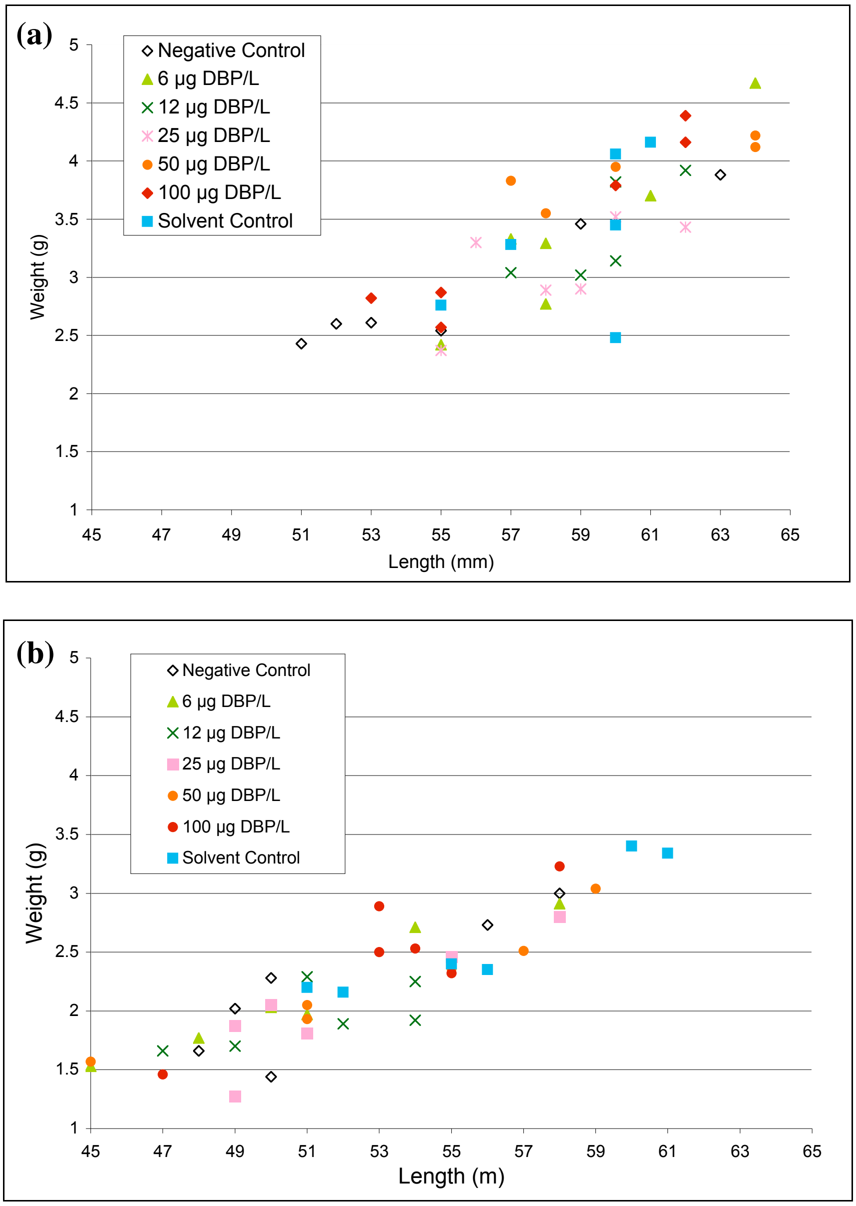


Figure 2.15. Length and weight of (a) male, and (b) female adult F_0 fathead minnows exposed to various concentrations of DBP for 21 days.

The 11-KT radioimmunoassay of male plasma samples was completed with only 10 of the 42 samples analysed had percent binding values too high (>82% binding) to be measured accurately on the linear part of the standard curve. Due to a lack of sample volume, these outliers could not be rerun, and thus their concentrations were extrapolated as conservative estimates. All other samples were considered to lie within the acceptable range for accurate measurement.

Overall, plasma 11-KT concentrations in F_0 males were found not to be significantly affected by DBP exposure (P=0.181, df.39, ANOVA) (Figure 2.16a). Interestingly, there was a slight, but non-significant trend of decreasing variability with increasing DBP concentration when the Negative Control data were omitted (P=0.06, N=34, Jonckheere-Terpstra Test). Further, if the data from all DBP-treated fish tanks were pooled, there did seem to be a slight, but non-significant, increase of plasma 11-KT in DBP-treated males compared to the Solvent Control (P=0.069, df. 33, ANOVA) (Figure 2.16b).

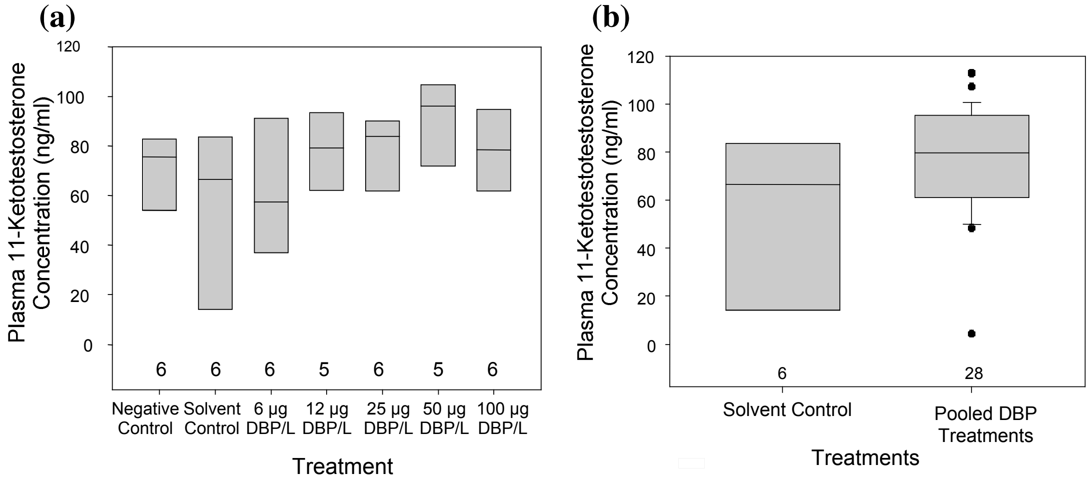


Figure 2.16. Box plots of plasma 11-KT concentrations of F_0 males that were exposed to various concentrations of DBP for 21 days in (a) all treatment groups, and (b) pooled DBP-treated groups compared to the Solvent Control only. (Boxes depict 25th and 75th percentiles abou the median; bars indicated the 10th and 90th percentiles and dots are outliers. The sample numbers are shown below each bar).

Male secondary sexual characteristics in the F_0 males failed to demonstrate any clear response to DBP concentration; both fat pad weight and tubercle number were not significantly different from the Solvent Control group at any exposure concentration (P>0.05, df.39, ANOVA)(Figure 2.17a and b). Fat pad index was also analysed with no evidence of an effect of phthalates on this parameter (P=0.448, df. 6, ANOVA on Ranks). Tubercle prominence also showed no response to DBP exposure (P=0.794, df. 39 ANOVA).

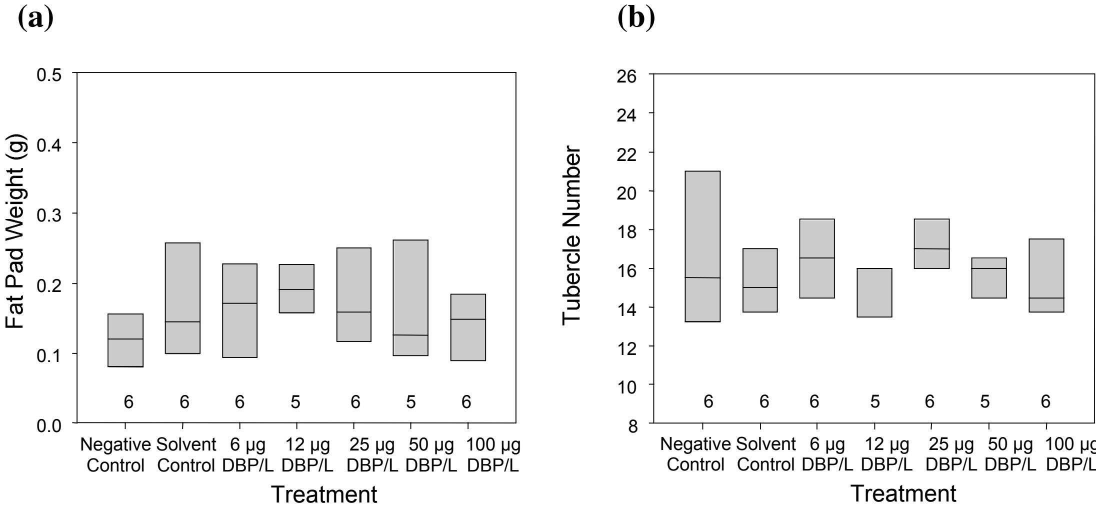


Figure 2.17. Box plots (25th and 75th percentiles surrounding the median) of (a) the fat pad weight, and (b) tubercle number, in F_0 males following a 21-day exposure to a range of concentrations of DBP. (Sample numbers are shown below each bar).

Histological analysis of the gonads revealed no identifiable evidence of DBP effects in either males or females. The ovaries of the females were all found to be at a similar stage of development, with the presence of mature vitellogenic oocytes in all samples (Figure 2.18). Oocyte atresia was observed in 3 females: one from the Negative Control group, one from the 12 μg DBP/L group, and one from the 100μg DBP/L group. While this could not be considered a concentration-response, the female with the most severe atresia (Grade 3 in severity, characterized by numerous atretic oocytes surrounded by normal oocytes) was identified in the highest DBP-treatment group (Figure 2.19).

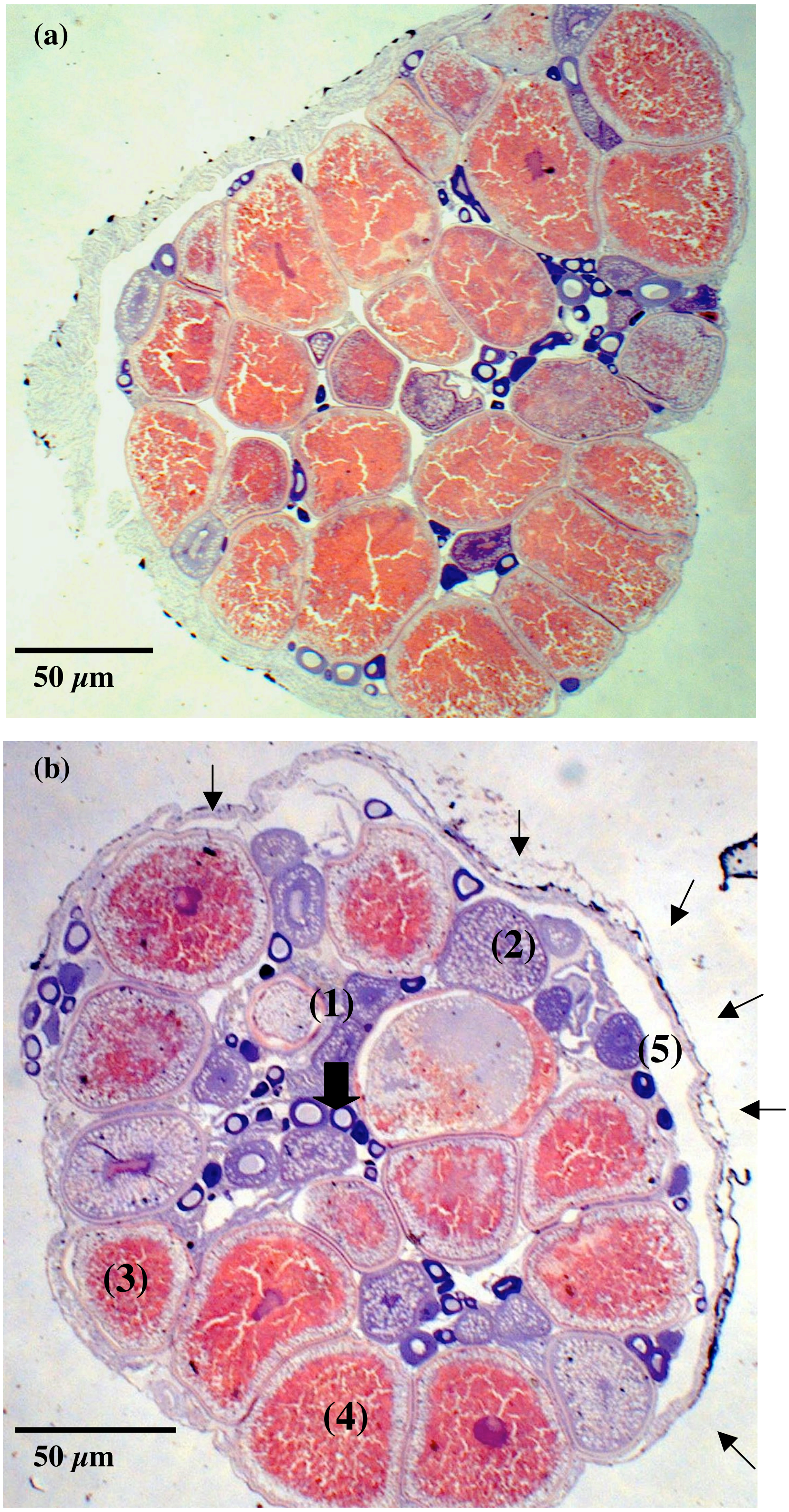


Figure 2.18. Sections through the ovaries of two F_0 female fathead minnows from the (a) Solvent Control group, and (b) 25 μg DBP/L group; (both at 20x magnfication). Cells indicated in (b) are (1) perinucleolar oocyte, (2) cortical alveolar oocyte, (3) early vitellogenic oocyte, (4) late vitellogenic oocyte ready to ovulate, and (5) is the ovarian cavity surrounded by the columnar ovarian cavity wall (indicated by small arrows).

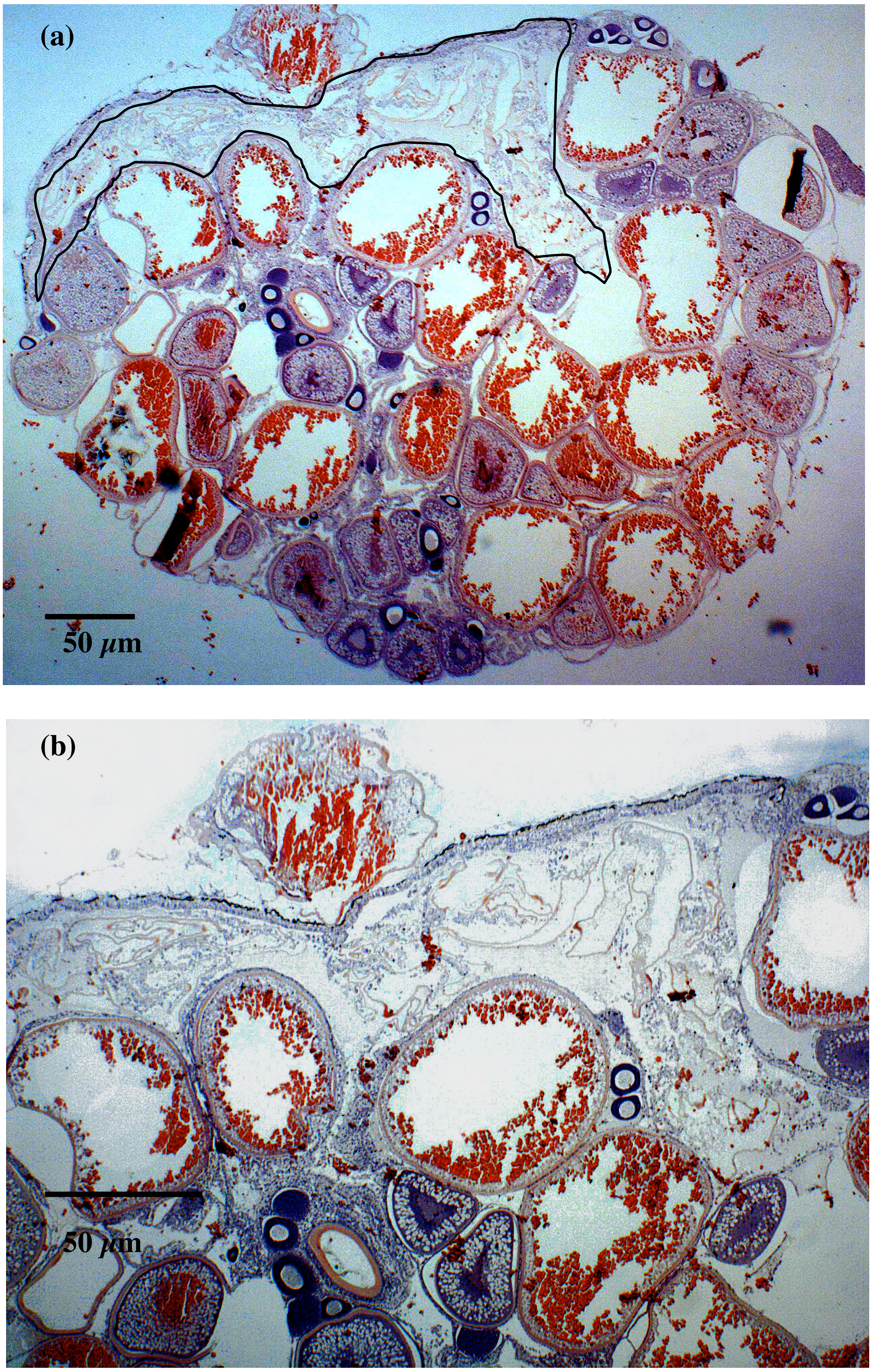


Figure 2.19. Sections through an ovary of a F_0 female fathead minnow from the 100 μg DBP/L tank. (a) The ovarian cavity is full of atretic follicles (grade 3 severity) demarcated by an outline (20x magnification). (b) The atretic area is enlarged at 40x magnification, showing clumped egg chorions, and accumulated yolk debris.

The F_0 males were also consistently mature, all producing spermatozoa. Ovo-testis was not observed, nor were any other abnormalities noted (Figure 2.20).

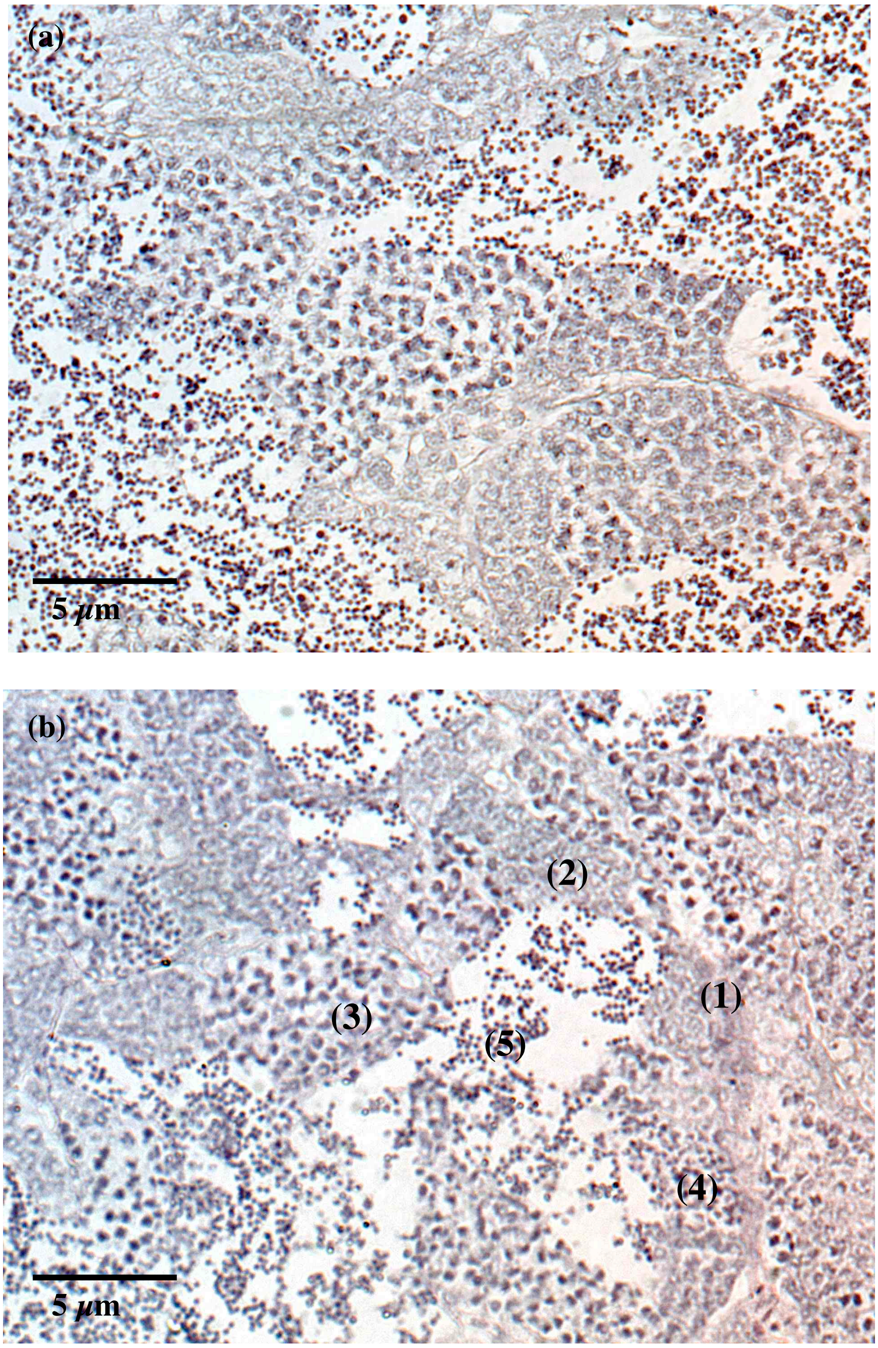


Figure 2.20. Sections of the testes from F_0 male fathead minnows from (a) the Solvent Control and (b) the 100 μg DBP/L group following a 21-day exposure period (400x magnification). Cell types are marked in (b) as, (1) spermatogonia, (2) spermatocytes, (3) primary spermatids, (4) secondary spermatids, and (5) spermatozoa in the lumen.

Fecundity was analysed in various ways in order to determine if DBP had any effect on spawning frequency or egg production. Spawning frequency was assessed by analysing the cumulative number of spawning events in each tank over time (Figure 2.21). Interestingly, fish in the two highest concentration groups, 50 and 100 μg DBP/L, took the longest period to begin spawning, on Days 6 and 11, respectively. Survival analysis measured no statistical differences in the timing of spawning between any of the treatments during the 21-day experiment, although only marginally (P=0.060, Kaplan-Meier Survival). Frequency was also analysed by averaging the total number of spawning events in each fish tank and dividing it by 21 days to yield the mean number of spawns per day. The mean number of spawns per day did not appear to be reduced by exposure to DBP, as there was no concentration-related response (Figure 2.22a). The mean number of viable eggs per spawning event also had no clear relationship with DBP treatment (P=0.073, df. 6, ANOVA on Ranks) (Figure 2.22b).

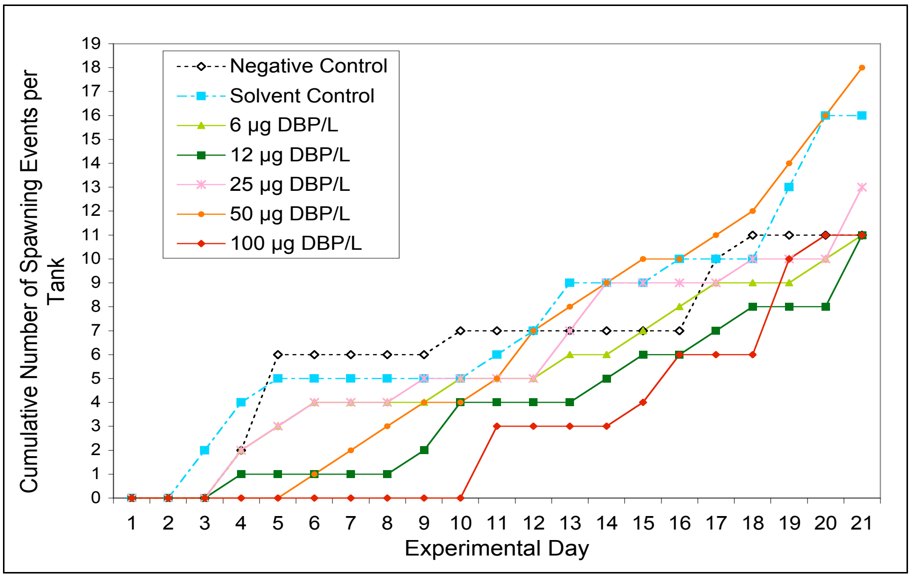


Figure 2.21. Cumulative number of spawning events of the F_0 fathead minnows in each treatment over 21 days.

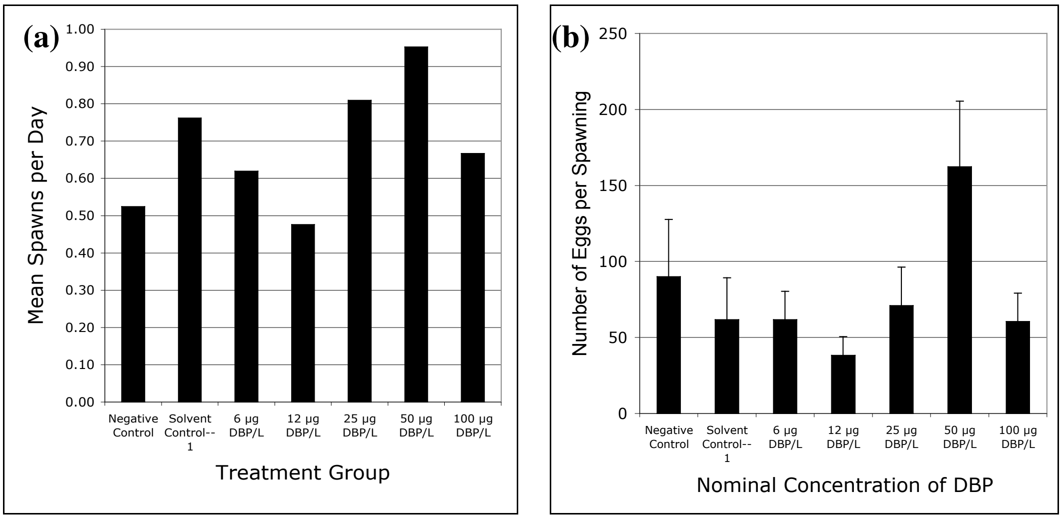


Figure 2.22. (a) The mean number of spawns per day in each treatment tank, and (b) the mean number of eggs per spawning event (± SD) in each tank of fish exposed to various concentrations of DBP over a 21 day period. There were six breeding pairs per tank.

2.3.4 F_1 Fish: Early sampling

The survival rate of the fry from embryos to 100 dph across all tanks was very low (<32% in all tanks) (Table 2.6). This mortality was thought to occur mainly prior to hatching due to fungal growth on the embryos. For example, the lowest survival rate was found in the group exposed to the highest DBP concentration and the highest survival was in the second highest DBP concentration group.

Table 2.6. Percent survival and numbers of F_1 fish that survived to 100 dph while being exposed to various concentrations of DBP.

Treatment	Number of fertilized eggs added	Number of fish at 100 dph	Survival (%)
Negative Control	552	85	15.4
Solvent Control—1	230	40	17.4
Solvent Control—2	990	144	14.5
6 μg DBP/L	395	125	31.6
12 μg DBP/L	572	82	14.3
25 μg DBP/L	363	57	15.7
50 μg DBP/L	1058	254	24.0
100 μg DBP/L	623	55	8.8

Histological examination of the gonads of the F_1 generation fish at 100 dph revealed that ovaries of the females were fairly variable in terms of developmental stage. The ovaries of all fish contained vitellogenic oocytes ranging from Stage 1 (mostly pre-vitellogenic follicles) to Stage 4 (mostly late vitellogenic follicles) (Figure 2.23). The ovaries of the Negative Control females were the least mature, found to have reached Stage 1, but the Solvent Control—2 group was found to have females with ovaries at Stages 1, 2 and 3. The only DBP-treated group with females with Stage 4 ovaries was the highest concentration, 100 μg DBP/L. However, there was no clear concentration-related response of developmental stage to DBP exposure in the female ovaires. Again, oocyte atresia was noted in 3 fish: one in each of the Negative and Solvent Control groups, and one in the 25 μg DBP/L treatment group.

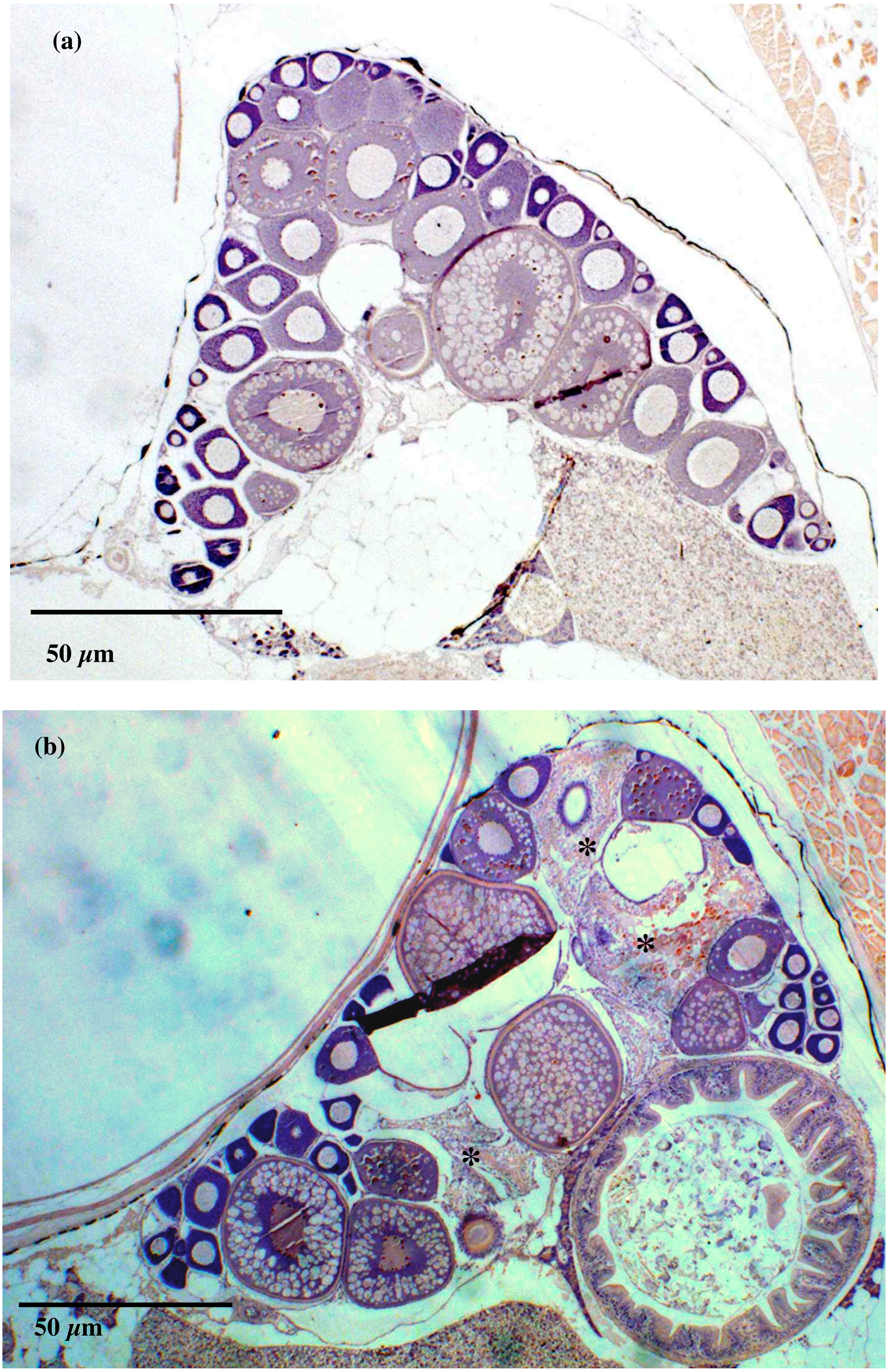


Figure 2.23.—see caption below

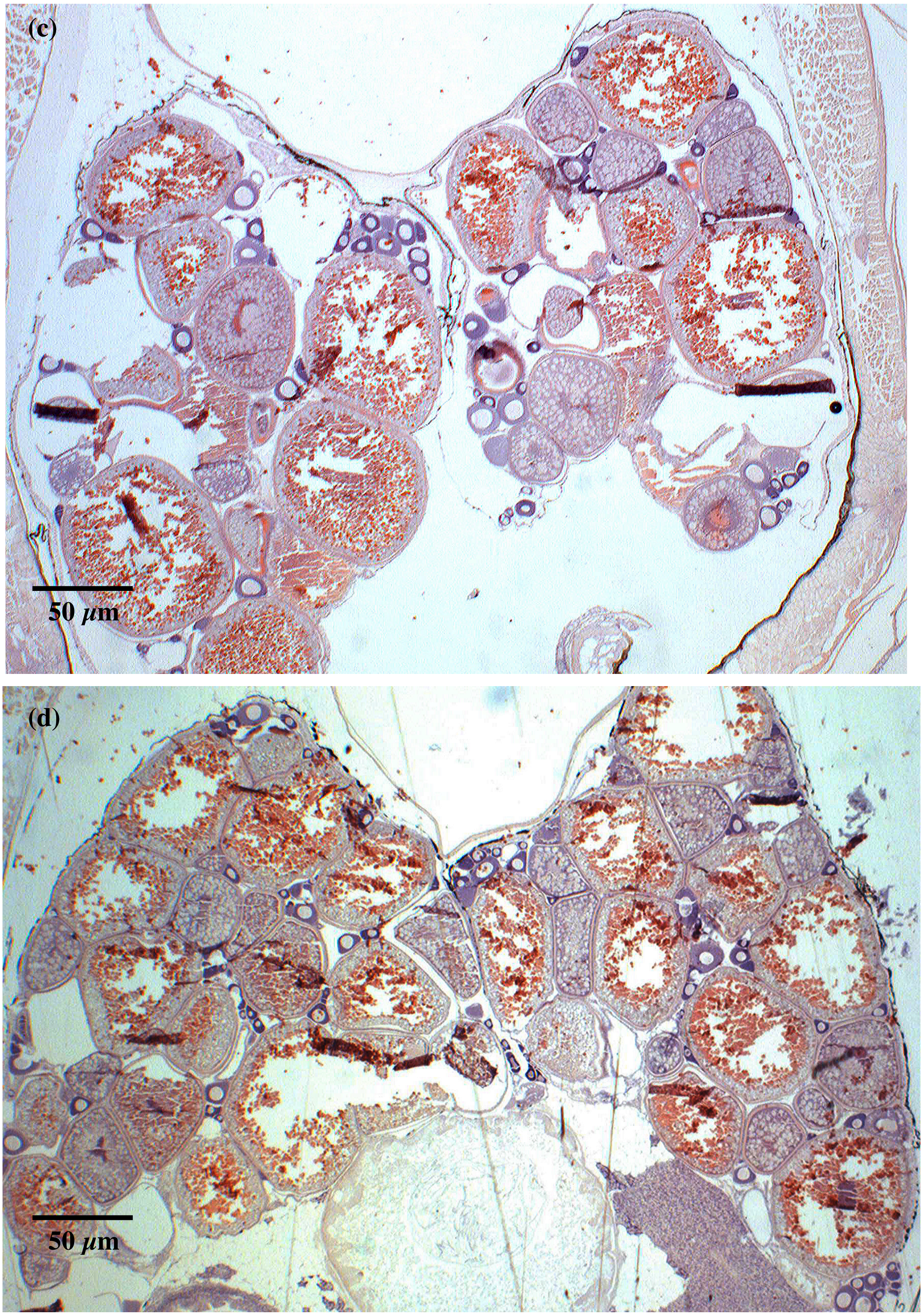
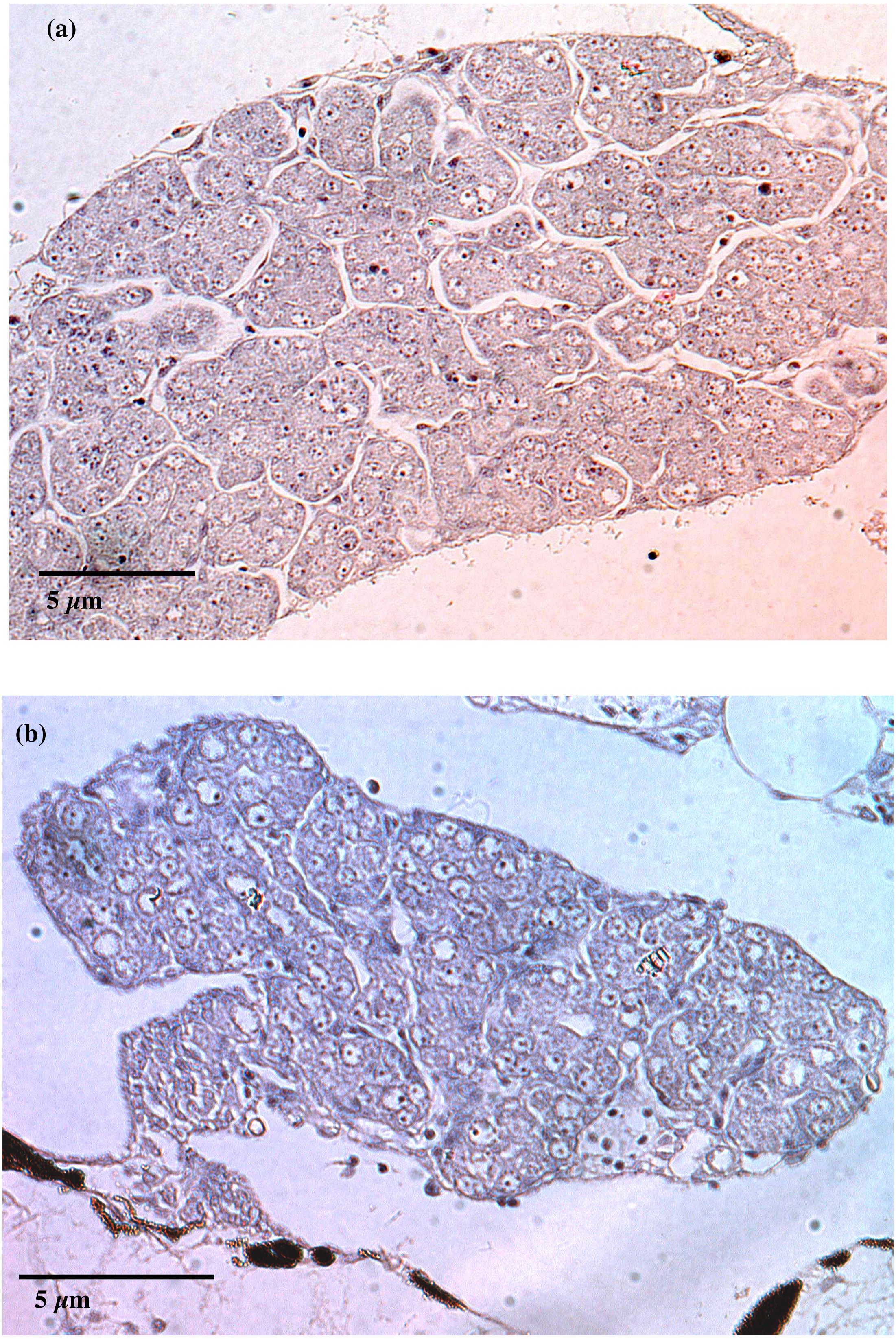


Figure 2.23. Whole-body cross sections of F_1 females showing the variation in gonadal development at 100 dph in (a) Negative Control with ovaries at Stage 1, (b) 25 μg DBP/L with ovaries at Stage 1 (* atretic oocytes), (c) Solvent Control—2 with ovaries at Stage 3, and (d) 100 μg DBP/L with ovaries at Stage 4. (All photographed at 20x magnification. Sections (c) and (d) are of poor quality due to the later stages of the oocytes, which are very hard and difficult to section without tearing).

The samples from the male fathead minnows at 100 dph were also fairly variable in terms of the developmental stages of their testes. The testes of the majority of the males (14 of 19) had only reached the juvenile stage, with testes containing only spermatogonia (Figure 2.24 a and b). The testes of one male in the Solvent Control group, two males in the 12 μg DBP/L group, and one in the 50 μg DBP/L group, had reached Stage 0, with the appearance of later phases of spermatogenesis up to spermatids, but with no spermatozoa (Figure 2.24c). Only one fish, from the 12 μg DBP/L group, was recorded with testes at Stage 1, with the appearance of spermatozoa in small proportions (Figure 2.24d). No abnormalities, such as ovo-testis or changes to the amount of spermatogonia, were noted.



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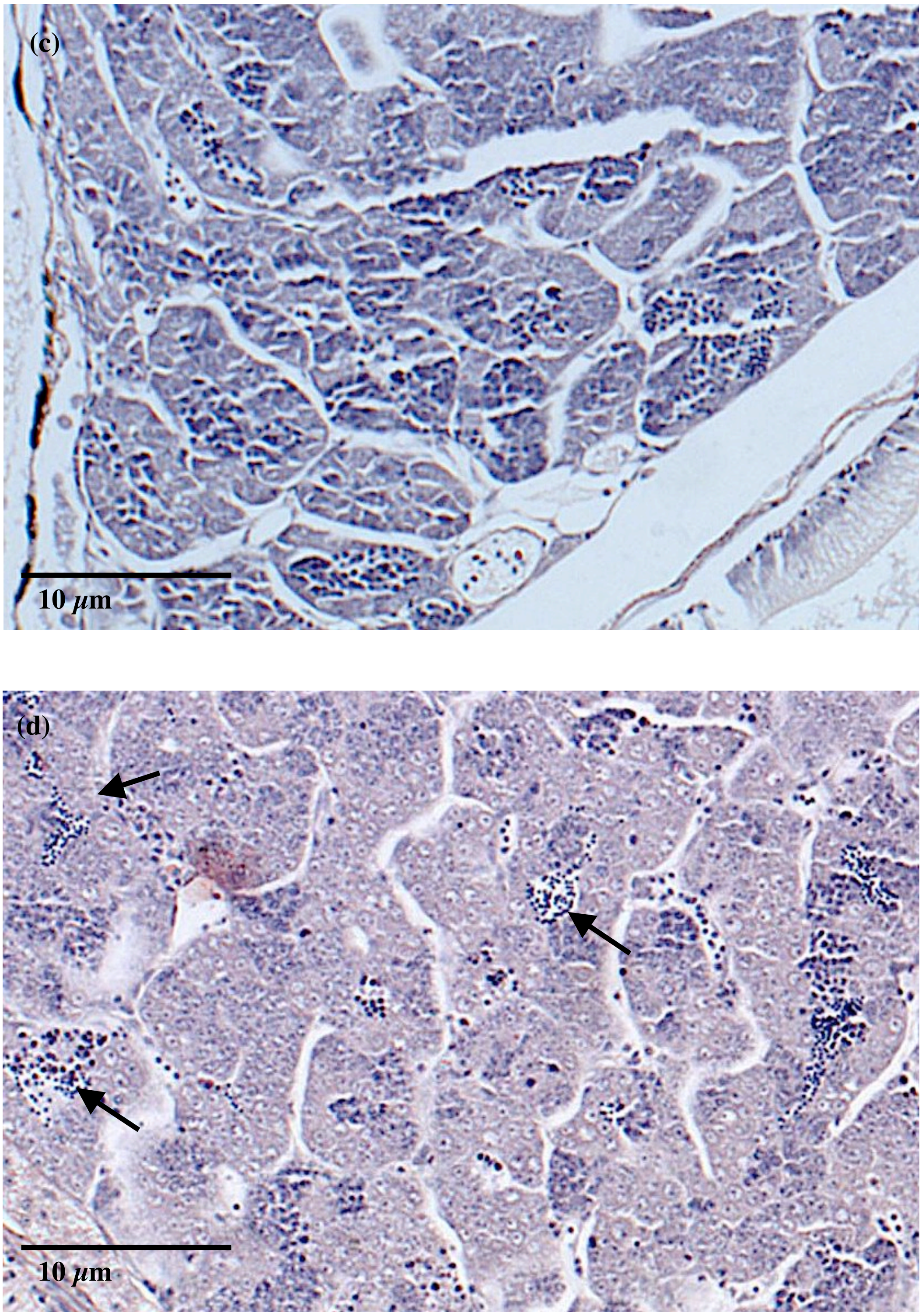


Figure 2.24. Sections of the testes of F_1 males, showing the variation in development at 100 dph. The fish are from (a) Solvent Control—2 group with testes at the Juvenile Stage, (b) 100 μg DBP/L group with testes in the Juvenile stage, (c) Solvent Control—2 group with testes at Stage 0, and (d) 12 μg DBP/L group with testes at Stage 1 showing mature spermatozoa in a lumen (arrows) (Images (a & b) are at 400x magnification, and (c & d) are at 100x magnification).

2.3.5 F_1 Generation: Adult sampling (150 days post-hatch)

The length and weight of the F_1 generation fish at 150 dph were analysed in males and females separately. Statistical analysis showed that both male and female fathead minnows from the Negative Control had significantly smaller lengths and weights when compared to fish from any other treatment tank ($P < 0.05$, df. 177 (male), df. 193 (female), ANOVA). This suggests that the fathead minnows in the Negative Control group did not experience equivalent growth conditions as the fish from other groups. This is probably because solvent use often leads to bacterial growth, which may provide extra food to the fish. The lengths and weights of the fish in the Solvent Control groups were not statistically significantly different from one another in any of the analyses ($P>0.05$, df. 42 (males), df. 51 (females), t-test) and thus were pooled.

Length did not appear to be related to DBP concentration in either male or females. However, significantly lower mean fork length was found in females exposed to 50 μg DBP/L, and males exposed to 12, 25, and 50 μg DBP/L compared to the Solvent Control groups ($P<0.05$, df. 177, ANOVA (males); df. 168, ANOVA on Ranks (females))(Figure 2.25a and b). In terms of weight, the female fish exposed to 50 μg DBP/L, and the male fish exposed to 12 and 50 μg DBP/L, were found to have significantly lower weight than those in the Solvent Control groups ($P<0.05$, df. 6, ANOVA on Ranks (males); df. 193, ANOVA (females))(Figure 2.25c and d). It was thought that perhaps differences in fish density between the various tanks were responsible for these length and weight differences. However, while the relationship between density and fish size was negative (Figure 2.26), it was not significant for either mean length or weight in males or females ($P>0.05$, df. 176 (male), df. 192 (female), Pearson Product Moment Correlation).

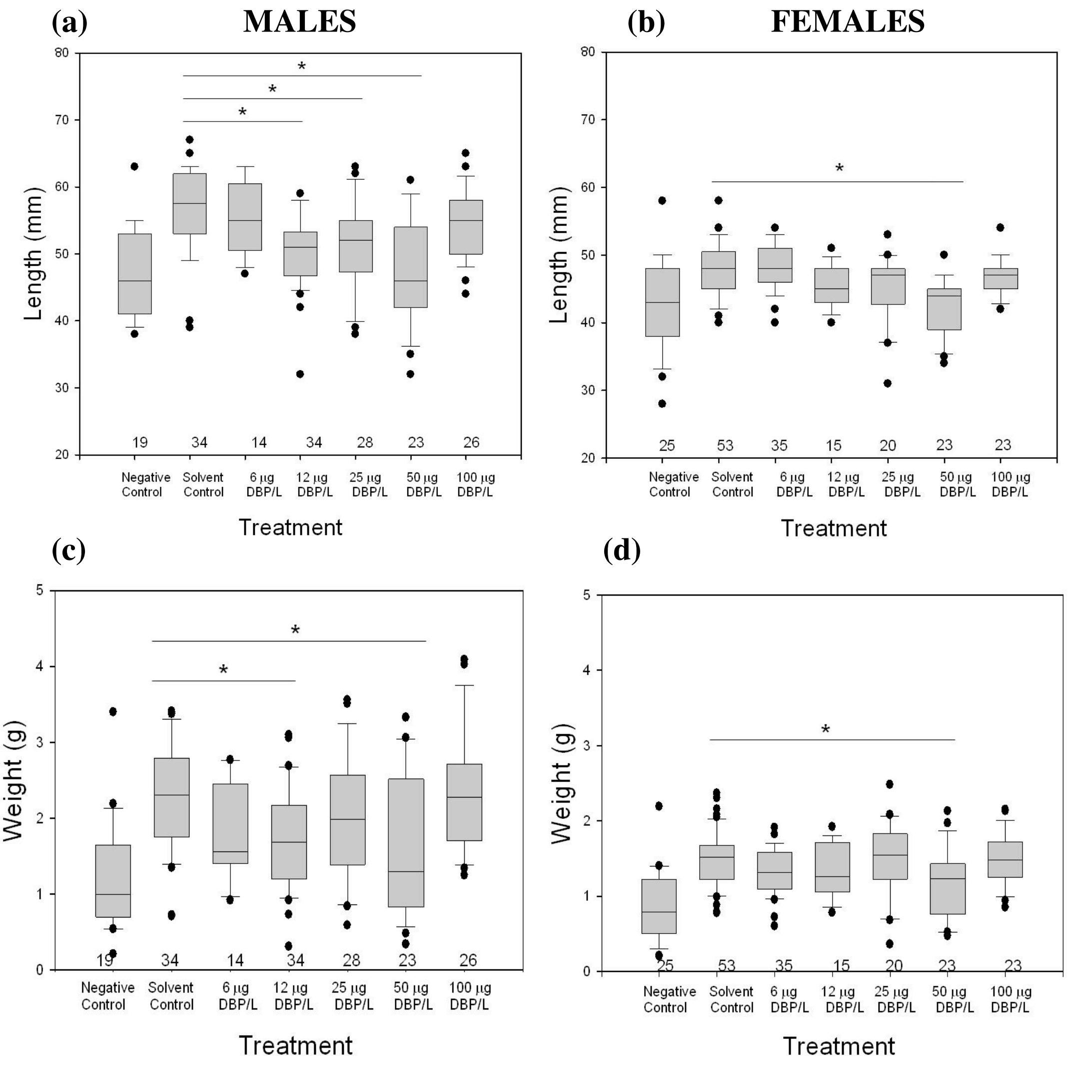


Figure 2.25. Box plots of length and weight of (a & c) males, and (b & d) females (* P<0.05 compared to the Solvent Control group. Boxes depict median with 25th and 75th percentiles. The bars extend to the 10th and 90th percentiles with outliers depicted as dots. Sample sizes are shown above each box).

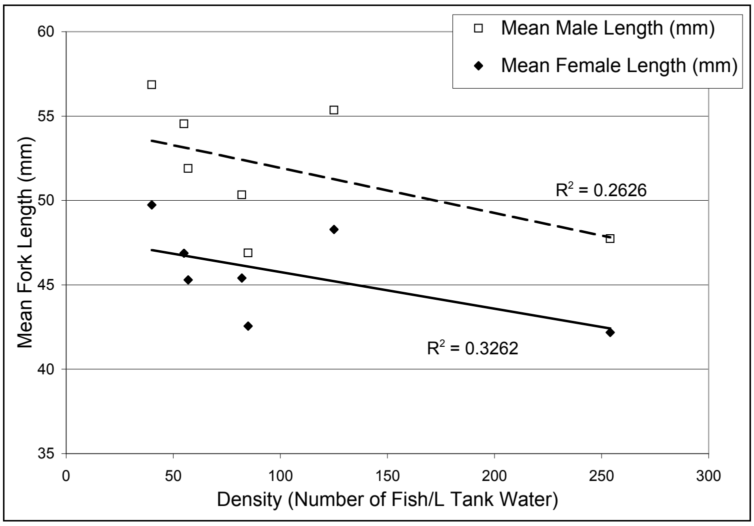


Figure 2.26. The relationship between the fish density in each tank and the mean fork length of fish in each treatment group from the F_1 generation at 150 dph. Males and females have been analyzed separately. Lines are linear regressions.

The male to female sex ratio in the Solvent Controls was 0.95 ± 0.58, and in the pooled DBP treatments was 1.12 ± 0.26 (mean ± SEM). Despite these values, the male to female ratio was fairly variable, this was unexpected considering the large sample sizes exceeded n=38 in all groups. However, when compared to the Solvent Control—1 and 2 groups, this response appeared to fall mainly within the same range of variation. Thus, it was not likely DBP-related and was also not significant (P=0.94, df. 6, χ^2) (Figure 2.27).

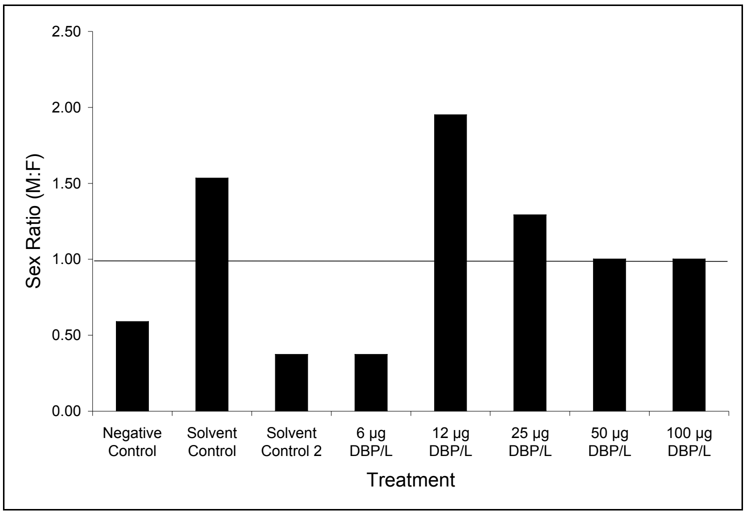


Figure 2.27. Sex ratios in each treatment group of F_1 fathead minnows that were exposed to various concentrations of DBP sampled at 100 and 150 dph.

The 11-KT concentrations in the F_1 males from the Solvent Controls were not statistically significantly different from one another, but this was marginal (P=0.057, Mann-Whitney Rank Sum Test). Thus, the Solvent Controls were not pooled.

The average concentrations of 11-KT in the plasma of F_1 males treated with DBP concentrations of 12, 25, 50, and 100 μg/L were significantly higher than the average 11-KT concentration in the Solvent Control—1 group (P<0.001, df. 7, ANOVA on Ranks)(Figure 2.28). This significance was also noted when the Solvent Control groups were pooled (P<0.001, df. 6, ANOVA on Ranks).

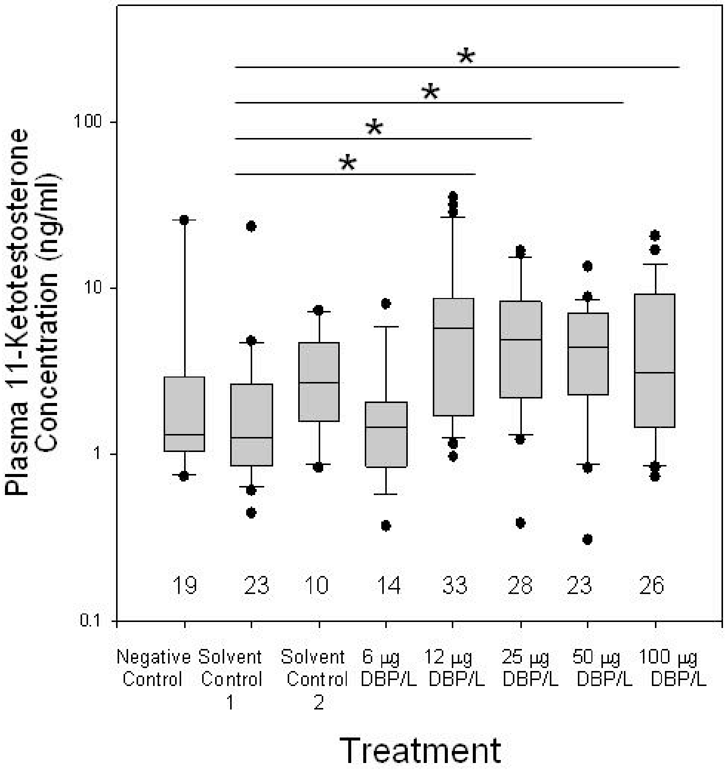


Figure 2.28. Box plot of plasma 11-KT concentrations on a log scale from F_1 male fathead minnows exposed to various concentrations of DBP (*P<0.05 compared to the Solvent Controls. Boxes indicate 25th and 75th percentiles about the median, bars indicated 10th and 90th percentiles with dots as outliers. Sample sizes are shown below each bar).

Histological examination of the gonads of F_1 generation fish at 150 dph showed no observable changes in response to DBP exposure. The gonads of both male and females in all groups had reached maturity and were producing spermatozoa and mature oocytes, respectively. No incidences of ovo-testis were observed. Females were found to have well-developed ovarian cavities with a columnar appearance (Figure 2.29). Similarly, the reproductive ducts of all males appeared normal, with a single point of attachment to the body wall (Figure 2.30a).

Unfortunately, the tissues of several male fish appeared to have been poorly fixed during histological processing. The poorly fixed samples were characterized by areas of testicular tissue with cells that appeared pale and fragmented (Figure

2.30). Initially, these abnormal cells were thought to be related to DBP exposure, since they were similar in their appearance to the dysgenic seminiferous tubules found in male rats exposed to phthalates (see Chapter 1, Figure 1.3). However, these abnormal tissues were later identified as post-mortem autolysis resulting from improper tissue fixation (Dr. Jeff Wolf, personal communication, Experimental Pathology Laboratories in Sterling, Virginia, USA). It is likely that the volume of the fish to Bouin's fixative used in the current study was higher than is required for the proper preservation of the gonads (usually 1:20). Since the testes are particularly sensitive to this effect, several samples across all treatments were affected. There were no statistically significant differences between treatment groups when the area of dysgenesis per testis area or the number of fish in each treatment group with dysgenesis were analysed ($P>0.05$, df. 6, ANOVA on Ranks).

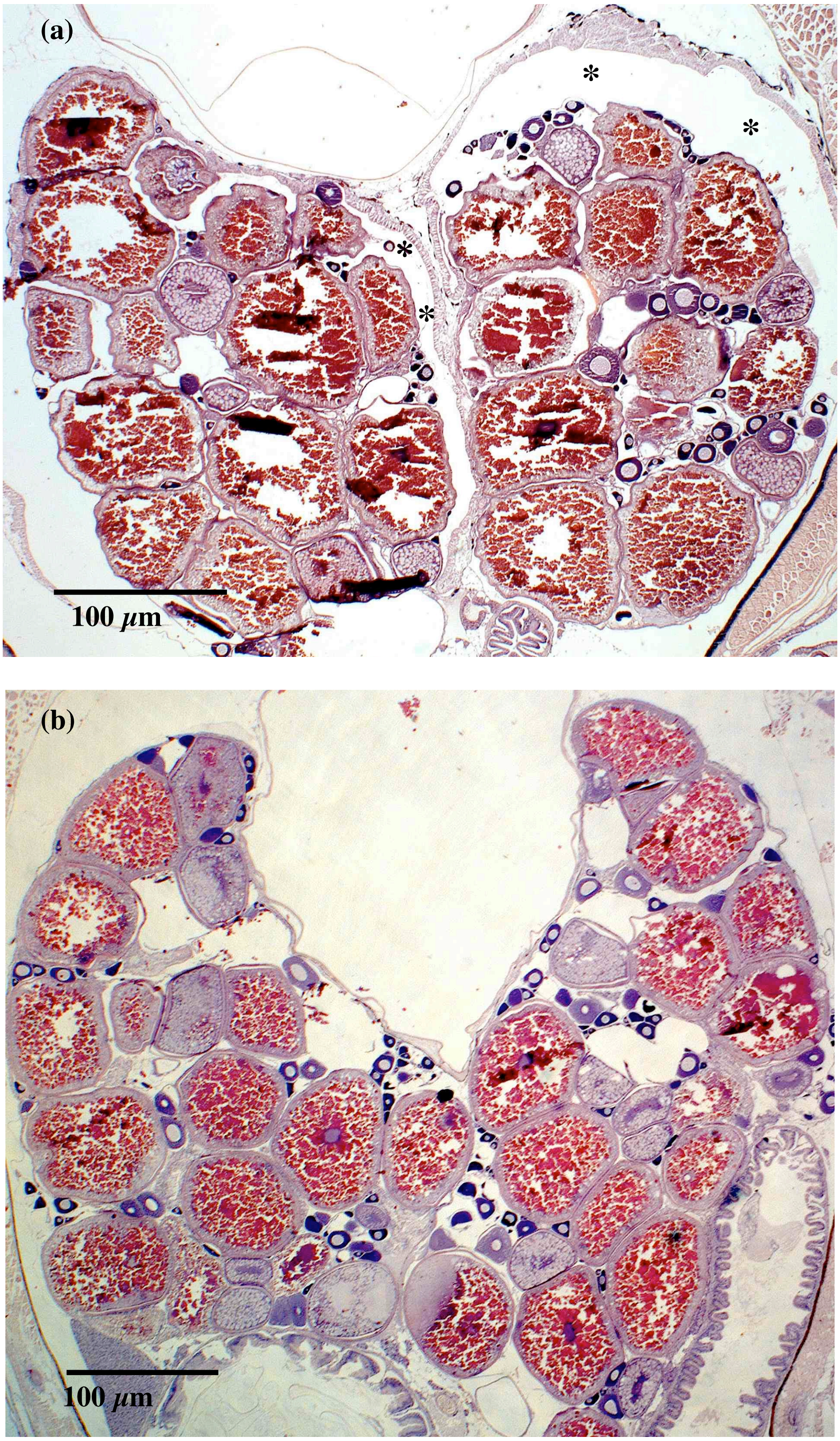
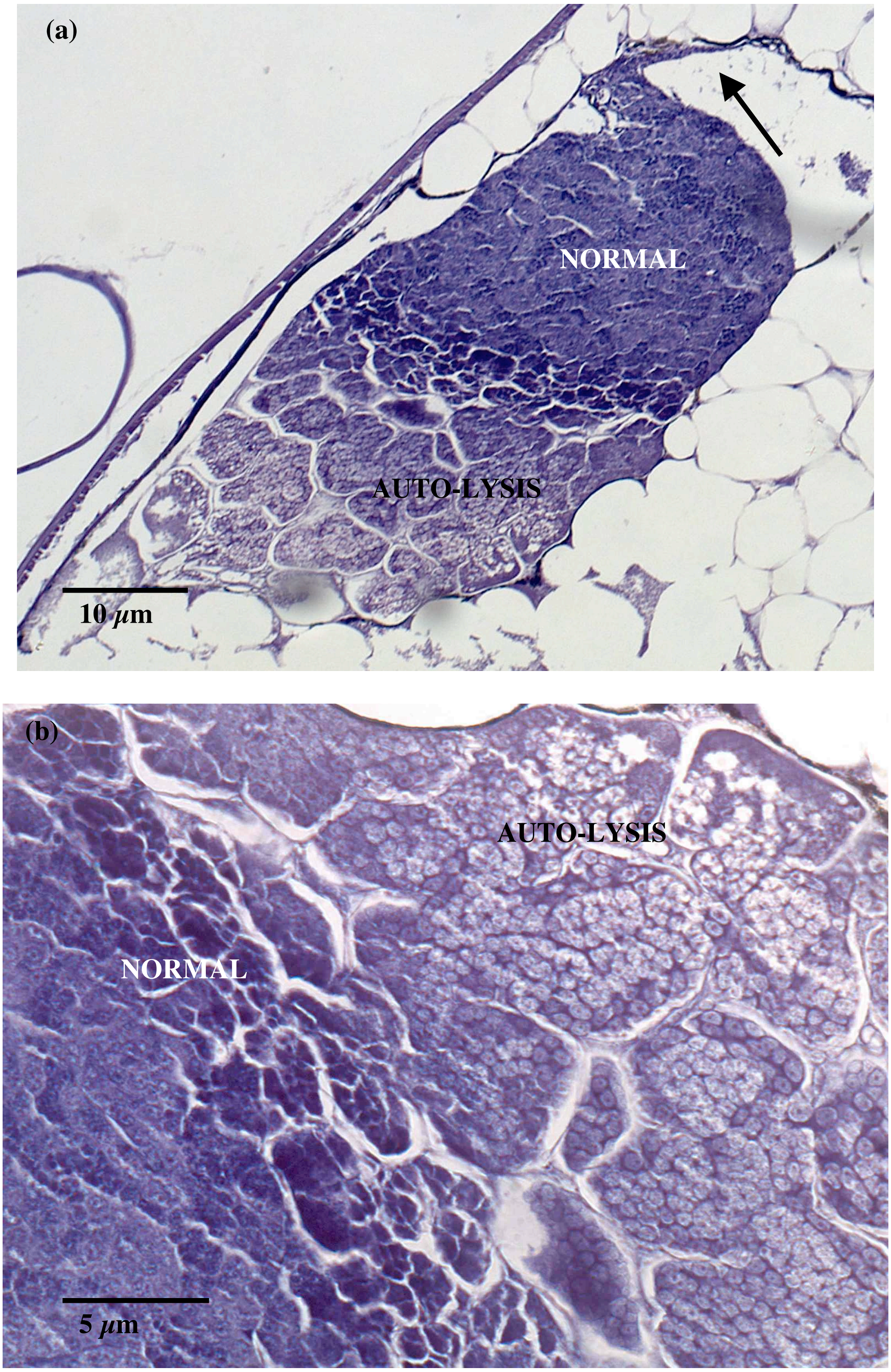


Figure 2.29. Photographs of ovaries of F_1 females in cross section from (a) the Solvent Control group with an * marking the ovarian cavities, and from (b) the 12 μg DBP/L group, both exposed from 0-150 dph (20x magnification).



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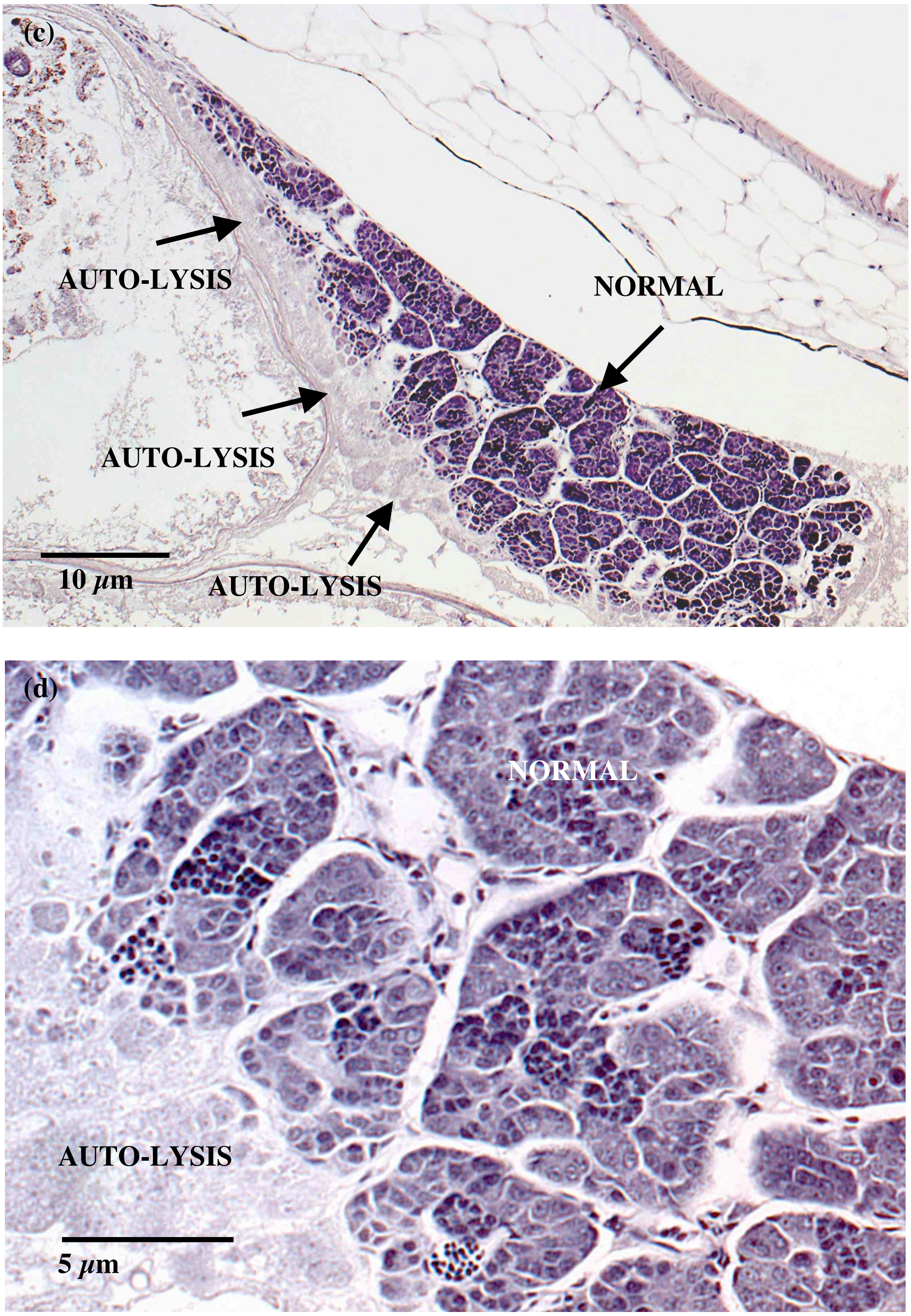


Figure 2.30. Photographs of the testes of F_1 males at 150 DHP from the (a) Negative Control group with the single point of attachment shown with an arrow (100x magnification), and (b) at 400x magnification, and from the (c) 12 μg DBP/L group at 100x magnification and (d) at 400x magnification. The areas of properly preserved testicular tissue are marked as "Normal", while the tissues that underwent post-mortem auto-lysis due to improper preservation are marked "Auto-lysis".

2.4 Discussion

2.4.1 Analytical chemistry

Clearly, there were problems encountered with various aspects of this study. However, the results did manage to address some of the questions surrounding the potential anti-androgenic activity of DBP in fish.

The analytical water chemistry failed to confirm that F_0 and F_1 fathead minnows were exposed to concentrations of DBP similar to the nominal concentrations (Figure 2.13). Instead, mean measured concentrations of DBP were approximately 40 to 50% of the nominal values. While these losses are commonly encountered in aqueous exposure studies (Ankley *et al*. 2007; Harries *et al*. 2000), it remains unclear whether or not the measured DBP concentrations represent actual concentrations of DBP in the fish tanks, or are underestimations due to poor analytical techniques.

Possible explanations for the low recovery of DBP from the tank water include:

i) The carrier solvent (DMF) was unable to properly dissolve and deliver the DBP into the water.

ii) The DBP was degraded in the tank water by bacterial metabolism or light.

iii) The DBP adsorbed to surfaces, such as the tank walls or the tubing.

iv) The DBP was taken up by the fish to an extent that its concentration was reduced in the water.

v) The analytical techniques were inappropriate:

  - The DBP may have degraded in the water samples between sample collection and extraction.

- Reverse-phase solid-phase extraction may have been a poor extraction method, leaving much of the DBP in the water sample.
- Hexane may have been too lipophilic a solvent to properly elute the DBP from the SPE cartridge.

Thus, there are several possible reasons why the DBP concentrations in the fish tanks may actually have been much higher than our results suggest. Overall, due to the pronounced variability of DBP concentrations within each treatment tank, the absence of an internal standard (such as deuterated DBP) with which to validate the analytical procedure, and the long time-lag between water sampling dates, it is very difficult to determine whether or not the fathead minnows were exposed to DBP concentrations in a consistent way. However, in looking at the difference in the measured concentrations between each tank over the course of the experiment (Figure 2.13), it is apparent that at each sampling date a range of distinct DBP treatments was achieved. This is further supported by the average concentrations of DBP, which also suggest that the treatment tanks spanned a gradient of concentrations, approximately half of their nominal values (Table 2.5). Thus, it can be argued that the fathead minnows in each of the treatment tanks represent groups of fish exposed to treatment concentrations distinct from each other.

Considering that:

(i) DBP stock solutions were physically pumped into the fish tanks in a consistent manner.

(ii) The chemistry results from this study suggest that the fathead minnows were exposed to a range of DBP concentrations.

(iii) Chemistry results obtained from subsequent exposure studies have shown that DBP can be administered to fish tanks in a flow-through system in a consistent manner.

It is likely that the fathead minnows were exposed to a range of DBP concentrations. However, assumptions beyond this would be irresponsible, especially given that the chemical techniques employed were not properly tested beforehand. The biological results therefore, represent probable responses to DBP, but we cannot definitively conclude these responses are true reflections of DBP exposure.

2.4.2 Effects of DBP on F_0 and F_1 generation fathead minnows

2.4.2.1 Length and weight

DBP did not appear to affect the length and weight of the F_0 generation fish (Figure 2.15). However, in the F_1 generation at 150 dph, some differences were apparent (Figure 2.25). In this case, fish in the Negative Control group tended to be much smaller in length and weight than fish in any other treatment tanks, including the Solvent Control. This was probably due to the increased bacterial growth in the Solvent Control and DBP-exposed tanks, which may have provided an additional source of food for the growing juveniles.

While not consistent, some of the groups of F_1 DBP-treated fish sampled at 150 dph were smaller in length and/or weight, sometimes significantly so, compared to those in the Solvent Control (Figure 2.25). However, there did not appear to be any consistent pattern of DBP concentration related to length and/or weight in either the male or female fathead minnows sampled at this time. Similarly, it is unlikely that stocking density affected lengths and weights of the F_1 generation fish at 150 dph, as there did not appear to be a particularly strong relationship when plotted against length (Figure 2.26) or weight (data not shown). Overall, it appears that DBP did not have a strong impact on fish length and/or weight in either F_0 or F_1 generations of fish.

2.4.2.2 GSI in F_0 fathead minnows

There appeared to be no effect of DBP on GSI of either F_0 males or females (Figure 2.14). While it is still possible GSI could be affected by DBP exposure in fathead minnows, it is more likely that DBP has very little if no effect on this parameter. For example, in mammals, phthalates only affect the GSI of rats at very high doses (>500 mg DBP/kg/day) (Gray *et al.* 1977; Mylchreest *et al.* 1999). A previously conducted 6-week pair-breeding test on fathead minnows found no effect of 69 and 82 μg BBP/L on GSI (Harries *et al.* 2000). Other reports involving dietary exposure of F_1 zebrafish, exposed to phthalates from egg to maturation, have also noted a lack of an effect of DINP and DIDP on the GSI in the adult fish (Patyna *et al.* 2006). In female fathead minnows, the single most important factor affecting the GSI is considered to be spawning interval (Jensen *et al.* 2001). However, significant reductions in the GSI of females were reported following a 90-day exposure of Japanese medaka to 10 and 50, but not 1, μg DEHP/L (n=12, 13 and 9, respectively*)* in an experiment using a static-renewal system. However, as previously discussed, the concentrations of 10 and 50 μg DEHP/L exceed the saturation concentration of this chemical in water, which is considered to be 3 μg/L. Further, while analytical chemistry was monitored in a tank with a nominal concentration of 50 μg DEHP/L for 72 hours, there was no measurement of the actual exposure concentration in the fish tanks at any point during the experiment (Kim *et al*. 2002). Thus, the effect of DBP on female GSI remains in question.

2.4.2.3 Secondary sexual characteristics in F_0 males

The secondary sexual characteristics of the F_0 males did not appear to be adversely affected in any of the DBP-exposed groups (Figure 2.17). A lack of an effect of BBP on such parameters has previously been reported in the fathead minnow (Harries *et al*. 2000). However, due to the proven anti-androgenic nature of DBP in mammals, and the questionable analytical results of this study, it remains a topic for further investigation.

2.4.2.4 Fecundity and spawning frequency

While this study was not conducted as a classical pair-breeding study, in which the ability to reproduce is assessed in distinct breeding pairs of fish, fecundity and spawning frequency did not appear to be significantly altered by DBP exposure. Interestingly, DBP-exposed fish appeared to have a delayed onset of spawning, although this was not statistically significant when analysed by Kaplan-Meier Survival Analysis (Figure 2.21). Spawning was observed in the Solvent Control tank after 3 days. Fish in the second highest concentration of 50 µg DBP/L took three days longer to spawn than the Solvent Control fish (on Day 6), while the highest exposure group of 100 µg DBP/L took the longest: eight days longer than fish in the Solvent Control tank (on Day 11). However, neither the average number of spawns per day, or the mean number of eggs spawned per day, appeared to be correlated to

DBP in a concentration-related manner (Figure 2.22). Thus, it seems that DBP did not have a strong negative effect on the fecundity of the fathead minnows.

The pair-breeding assay conducted by Harries *et al.* (2000) found that the frequency of spawning in fathead minnow pairs significantly decreased following BBP exposure during a 3-week exposure study. Interestingly, this decrease in frequency did not result in decreased fecundity, because the number of eggs per spawn also increased significantly. Similarly, exposure of zebrafish to a nominal concentration of 500 µg DBP/L in a static-renewal system for 15 days had no effect on egg number (Ortiz-Zarragoitia *et al.* 2006). It should be noted that a published abstract reported significant reductions in egg production of F_1 generation Japanese medaka exposed throughout their lives to dietary DBP at a concentration of 776 mg/kg/day (Patyna and Cooper 2000). However, this effect was found in F_1 fish, suggesting that effects on egg production may result from early life-stage exposure rather than adult-stage exposures, though this apparent effect was not replicated by the same laboratory using DINP and DIDP (Patyna *et al.* 2006).

While not significant, the finding of a longer period before first spawning in fish exposed to 100 µg DBP/L, when put alongside the finding of Harries *et al.* (2000), may be indicative of some effect of DBP on spawning. However, an actual pair-breeding study would be more appropriate to address these questions. Overall, I conclude that DBP did not appear to affect fecundity in any significant concentration-dependent manner in this study.

2.4.2.5 *Sex ratio of F_1 generation fathead minnows*

While the sex ratios were surprisingly variable (Figure 2.27), this did not appear to be related to DBP exposure as the Solvent Control—1 and 2 groups had a very high and very low sex ratio respectively. In other words, with the exception of the 12 μg DBP/L group, the range of sex ratios in the DBP-exposed groups were closer to 1:1 than the sex ratios of the two Solvent Control groups. This apparent lack of an effect is supported by the work of Norman *et al.* (2007) in which Atlantic salmon (*Salmo salar*) exposed to dietary concentrations of 400, 800, and 1500 mg DEHP/kg for 0-4 weeks post-hatch, had comparable sex ratios to control fish. Other studies have also found no concentration-related effects on the sex ratios of Japanese medaka exposed to DEHP during early life (Chikae 2004; Chikae *et al.* 2004).

2.4.2.6 Gonadal histology

The effects of phthalates on gonadal histology of the fathead minnow was a point of particular interest, because of the well-documented effects of these chemicals in mammalian gonads (see Chapter 1, Section 1.3.3). In the current study, very few, if any, histological anomalies were found in the gonads of either F_0 or F_1 generation fish (Figures 2.18, 2.20, 2.23, 2.24, 2.29, 2.30). Further, interpretation of the histological results was complicated by the inadequate fixation of the tissues in the samples from the F_1 generation at 150 dph (Figure 2.30).

Overall, it appeared that the only abnormality noted was oocyte atresia (Figure 2.19), observed in three F_0 females: one from each of the Negative Control, 12, and 100 μg DBP/L groups, and in three F_1 females sampled at 100 dph: one from each of the Negative Control, Solvent Control, and 25 μg DBP/L groups. Oocyte atresia was not noted in any of the F_1 females sampled at 150 dph.

Oocyte atresia has been noted previously in studies of the effects of phthalates on female fish, as well as in female rats exposed to phthalates in adulthood (Grande *et al.* 2006). The exposure of Japanese medaka to 0, 1, 10, and

50 μg DEHP/L from hatch to maturity led to a significant reduction in the number of mature oocytes in the groups of fish at the 10 and 50 μg DEHP/L concentrations (0% and 22% of females had mature oocytes compared to 54% in controls), while males were found to be unaffected (Kim *et al.* 2002). However, we must recall that this experiment was conducted in a static-renewal system without verification of the phthalate concentrations in the fish tanks themselves, and with nominal concentrations 3 and 17 times higher than the saturation point of DEHP.

Oocyte atresia has also been noted in female fish following exposure to other anti-androgens, such as vinclozolin (at 5000 μg/L) exposed for 21 days as adults, and flutamide (at 500 μg/L) exposed from 0-3 months post-hatch (Jensen *et al.* 2004; Kiparissis *et al.* 2003). As in the current study, atresia was only identified visually and was not confirmed by immunohistochemistry. Further, these authors also observed atresia in the controls, but at lower frequencies. In the case of Jensen *et al.* (2004) the incidence of oocyte atresia was concentration-related, but it was not in the flutamide exposure (Kiparissis *et al.* 2003). By contrast, another study, using vinclozolin, found no significant occurrences of oocyte atresia in female fathead minnows exposed to 200 and 700 μg vinclozolin/L from 6-40 dph (Makynen *et al.* 2000).

Overall, the relationship linking DBP to oocyte atresia in the female fathead minnows is tenuous. On one hand, the finding in the current study, of the most severe oocyte atresia in an F_0 female exposed to the highest concentration of DBP suggests there may be a link. On the other hand, considering that the incidence of oocyte atresia was very low (3 of 42 F_0 females and 3 of 17 F_1 females at 100 dph), that it was also observed in the control groups, that it was not observed in any of the females sampled at 150 dph, and that in the F_1 females sampled at 100 dph it was observed more often in the control groups than a DBP-exposed group, suggests that it is not an effect of phthalate exposure. Further, it is important to note that female fathead minnows reared in the laboratory are commonly observed to have a higher than normal number of atretic oocytes at the late vitellogenic stage. This phenomenon is thought to be linked to stress or not being given an opportunity to spawn (Dr. Jeff Wolf, personal communication). Overall, this greatly undermines the link between phthalate exposure to oocyte atresia in exposed female fathead minnows.

In general, it is possible that the lack of any significant findings on the gonadal histology of either male or female fathead minnows exposed to DBP both as adults and during development, might be the consequence of the concentrations of DBP being too low to elicit an effect. In mammals, histological effects tend to be observed at doses of >500 mg/kg/day, following either *in utero* or adult exposure (Gray *et al.* 1977; Mahood *et al.* 2005; Mylchreest *et al.* 2002; Oishi 1985), although in one study, multinucleated gonocytes were documented at concentrations as low as 135 mg DEHP/kg/day, but not at 15, 45, 135, and 405 µg DEHP/kg/day (Andrade *et al.* 2006). While it is impossible to compare the concentrations used in dietary exposures of rats to the concentrations used in aqueous exposures of fish, it is possible that the concentrations of phthalates used in this study were simply too low to affect gonadal histology.

On the other hand, it is possible that we were unable to observe the effects of DBP on gonadal histology due to a lack of proper analytical techniques. Without the use of immunohistochemistry, it is difficult to discern any specific and subtle histological effects caused by phthalate exposure. Immunohistochemistry involves the use of antibody-antigen reactions to stain specific cells, such as Sertoli and

Leydig cells in males, or atretic follicles in females. The use of this technique was simply not within the scope of this project. This is unfortunate, as it might have elucidated some very interesting responses to phthalate exposure in the gonads of the fish that we were unable to identify. It is well-documented in rats that *in utero* exposure to doses of phthalates as low as 10 mg/kg/day leads to abnormal clustering and/or hyperplasia of the Leydig cells in the rudimentary testis (Lin *et al.* 2008). This effect is more commonly documented at doses of 500 mg/kg/day or more, and is also persistent into adulthood (Barlow *et al.* 2004; Mahood *et al.* 2005; Mylchreest *et al.* 2002). Thus, it is possible that DBP exposure did induce such changes in the fathead minnows. However, without any specific stains, this remains unknown.

Overall, the existing histological data in fish seem to suggest that phthalates may have no effect if fish are exposed after they have reached maturation. For example, the exposure of adult zebrafish to a nominal concentration of 500 μg DBP/L for 15 days in a static-renewal system appeared to have no histological effects on the gonads of either males or females (Ortiz-Zarragoitia and Cajaraville 2005; Ortiz-Zarragoitia *et al.* 2006). Similarly, DBP was found not to induce ovo-testis following dietary exposure at doses of up to 776 μg/kg/day in F_0 Japanese medaka, while F_1 fish in the same experiment were found to have non-significantly different rates of ovo-testis in males to the rates in the controls (although it is unclear how long the F_0 generation were exposed) (Patyna and Cooper 2000). Similarly, when newly hatched Atlantic salmon were exposed to DEHP from 0 dph to 4 weeks post-hatch, a low incidence (6 out of 202, so approximately 3%) of ovo-testis in fish exposed to the two highest dietary concentrations of DEHP (800 and 1500 mg/kg) was observed; this effect that was significant at the highest dose (Norman *et al.* 2007). Compared to these findings, we found no evidence of ovo-testis in any of the fish in our experiment, despite the fact that the F_1 generation were exposed during both their egg-phase and after hatch. However, we did not use such high concentrations of phthalate as the above-mentioned studies, and used an aqueous route of exposure rather than the dietary exposure route.

In general, the results of this study in combination with the prevailing literature in both mammals and fish seem to suggest that histological changes in fish gonads may be the result of exposure to phthalates at any developmental stage, but that such changes may occur with fairly low frequency, require immunohistochemical techniques for their detection, or higher concentrations of phthalate than those used in the current work. Consequently, considerably more research is needed to conclusively demonstrate the effects of phthalates on gonadal histology (if they do, indeed, occur).

2.4.2.7 11-Ketotestosterone concentrations in males

Finally, while DBP exposure did not cause DBP concentration-related responses in the 11-KT concentrations between treatment groups, there did appear to be a general trend of higher concentrations of 11-KT in the plasma of male fish in both the F_0 and F_1 generations exposed to DBP compared to the Solvent Control. This trend was only marginally non-significant in the F_0 males when analysed with the Jonckheere-Terpstra Test (P=0.069) (Figure 2.16). However, in the F_1 generation males, the plasma 11-KT concentrations were significantly higher in all of the groups exposed to concentrations of $\geq 12\ \mu g$ DBP/L compared to the Solvent Controls (Figure 2.28).

While phthalates are generally considered to lower plasma testosterone concentrations in mammals (Culty *et al.* 2008; Higuchi *et al.* 2003; Lee *et al.* 2009), both increases and decreases in androgens have often been reported immediately following phthalate exposure. It appears that, at lower phthalate doses, plasma testosterone concentrations in rats are often increased, whereas at doses of 500 mg/kg/day and higher, they are consistently reduced. For example, one study found that rats exposed *in utero* to DEHP concentrations of 10, 100, and 750 mg/kg/day, had increased testicular testosterone concentrations in the 10 mg/kg/day compared to the control group, but at the higher DEHP doses the testicular testosterone concentrations were lower, significantly so in the 750 mg/kg/day group (Lin *et al.* 2008). Interestingly, a similar study conducted in another laboratory found that rats exposed *in utero* to DEHP to doses of 10, 30, 100 and 300 mg/kg/day also had non-significantly higher testicular as well as plasma testosterone concentrations in the 10 mg/kg/day group compared to the control group. Again, at the doses of 100 and 300 mg/kg/day, both testicular and plasma testosterone concentrations were lower in the male rats compared to the control groups, but this was only statistically significant for testicular testosterone concentration in the 300 mg DEHP/kg/day group (Borch *et al.* 2006).

This lack of consistency appears to also occur when comparing plasma testosterone concentrations to *ex vivo* testosterone production in rats. *Ex vivo* testosterone production appears to be more commonly reduced following phthalate exposure, while the response of plasma testosterone concentrations is, as discussed, not as predictable. Perinatal exposure of male rats to 10 and 100 mg/kg/day DEHP (21 to 90 or 120 days old) caused significant increases in plasma testosterone concentrations in the phthalate-exposed groups, but significant decreases in testosterone production by the excised testes of the same animals (Akingbemi *et al.* 2004). Interestingly, another study in the same laboratory found that, on one hand, exposure of male rats to 10, 100 and 200 mg DEHP/kg/day from PND 21-35 or 35-48 resulted in significantly lower *ex vivo* testicular testosterone production, in a dose-related manner. Whereas, on the other hand, exposure to the same concentrations of DEHP for a longer period, 21-48 PND, caused a significant increase in *ex vivo* testosterone production (Akingbemi *et al.* 2001). Thus, testicular testosterone production, too, may not necessarily yield more consistent results than plasma testosterone concentrations. In the context of the current work, the development of an assay to analyse *ex vivo* testosterone production in the fathead minnows would have been very interesting, considering the finding of elevated plasma 11-KT concentrations in both F_0 and F_1 males. Unfortunately, this was not within the scope of this project.

In fish, only one *in vivo* exposure study examining effects of phthalates on the steroid hormones testosterone, 11-KT and oestrogen has been reported to date (Han *et al.* 2009) . This experiment involved exposing an unknown number of Common carp (*Cyprinnus carpio*) to 0, 5.5, 10.5, 15.5, and 20.5 mg DEHP/L in a recirculated system for 48 hours with duplicate tanks. Male and female blood samples were pooled in each treatment group for an unknown reason, and analysed by radioimmunoassay. The authors reported a strong concentration-related increase in the plasma concentrations of 11-KT and testosterone in the pooled samples of DEHP-treated fish compared to those from the Solvent Control. This was significant in the 15.5 and 20.5 mg/L concentration groups ($n=1$, $P<0.05$). However, it must be noted that water chemistry was not conducted to confirm exposure concentrations, and more importantly, that all concentrations far exceed the saturation concentration of DEHP in water, which is only 3 μg/L. It is possible that such responses are genuine; however, these data should be approached with some scepticism.

Overall, considering that we know very little of the actual anti-androgenic mechanism of action of phthalates, significant hormonal increases as those observed in the current work may represent actual responses to phthalates at lower concentrations, perhaps via negative and/or positive feedback loops (Kime 1998). Alternatively, this increase could be a result of Leydig cell hyperplasia, which has been commonly reported in mammalian studies, but was not confirmed in the current work (Barlow *et al.* 2004; Mahood *et al.* 2005; Mylchreest *et al.* 2002). In general, more research is needed to elucidate this effect.

2.5 Conclusions

This experiment shed some light on the ability of DBP to act as an anti-androgen *in vivo* in the fathead minnow. However, it was unable to provide conclusive evidence to support the hypothesis that DBP is anti-androgenic in fish. DBP did appear to alter androgen concentrations in both F_0 and F_1 generations of fathead minnow males, significantly so in the latter, but did not appear to result in reduced fecundity, disrupted gonadal development, or altered expression of the secondary sexual characteristics.

The pilot study, as a whole, brought to light several problems that must be addressed in order to conduct phthalate exposures in fish:

i. Sound analytical chemistry methods must be established in order to confirm exposure concentrations.

ii. The most accurate methods for detecting the potentially subtle effects of phthalates in fish must be identified, validated, and incorporated into subsequent experiments, since assessing the anti-androgenic effects of a chemical with an undetermined mechanism of action can be difficult.

Thus, the next steps for this project were to establish and optimize the analytical chemistry methods, as well as the reconsider the fathead minnow as a suitable species for anti-androgenic endocrine disruption, as it was possible that a more sensitive species might exist.

Chapter 3. Analytical chemistry: Establishment of a method for the measurement of aqueous concentrations of di-*n*-butyl phthalate.

3.1 Introduction

Following the difficulty encountered when trying to determine DBP concentrations in the pilot study (Chapter 2), the next necessary step became the establishment of a reliable method to quantify DBP concentrations in water. During the pilot study, several questions were raised which needed to be addressed in order to test whether or not phthalates cause anti-androgenic endocrine disruption in fish:

1. Can a solvent dissolve DBP without degrading it?
2. Can this solvent deliver DBP into water in a flow through system accurately and consistently over time?
3. Is the method for extracting DBP from water using solid phase extraction accurate and reproducible?
4. Is the GCMS a reliable instrument for the analysis of DBP samples?
5. Can the uptake of aqueous DBP by fish be established either directly or indirectly?

A series of experiments were developed in order to address these questions. They were answered over the course of 10 months, from May 2007 to March 2008.

3.2 Miscibility experiment

3.2.1 Introduction

The aim of the miscibility experiment, conducted on September 11th 2007, was to characterize the behaviour of DBP, both in water and in DMF, at concentrations of 100 μg/L, by simple observation. This concentration was chosen since it was the highest nominal concentration used in the pilot study (Chapter 2). We hypothesized that DBP would readily dissolve in water, both with and without a solvent, at a concentration of 100 μg/L. This hypothesis was based on its saturation point in water at 11.2 mg/L (Staples *et al.* 1997).

3.2.2 Methods

The miscibility experiment was divided into 2 parts:

i. The direct addition of 100 μg DBP to 1 L of water.
ii. The addition of a solution of DBP in DMF to water.

In the first part, 100 μg of DBP was added directly with a pipette to a litre of de-ionized water in a clean glass beaker at room temperature. This was then mixed and observed. In the second part, 5 mg of DBP was dissolved in 1 ml of DMF to give a solution of DBP at a concentration of 5 g DBP/L. A pipette was used to add 10 μl of this solution to 500 ml water, to give a final concentration of 100 μg DBP/L, which was subsequently observed for 1 hour.

3.2.3 Results

When the DBP was pipetted directly into the water, it appeared to float in small, oily bubbles on the surface of the water. Upon vigorous mixing with a stir bar, the DBP would visibly break up into smaller globules, but would again float to the surface once mixing ceased. These globules were still visible after 5 minutes of mixing, and even after the water was heated to approximately 60°C and mixed for 30 minutes. In the second part, when the DBP was first dissolved in DMF solvent, the phthalate appeared to dissolve immediately in the water. No oily bubbles were detected.

3.2.4 Discussion and Conclusions

A review of several studies estimated that, based on octanol/water partitioning, DBP is soluble in water at concentrations up to 11.2 mg/L (Staples *et al*. 1997). In practice, this appears to be much more difficult to acheive. It is possible, for example, that the solvent provides a medium in which to disperse the DBP, so that when it comes into contact with the water, it is more easily dissolved. However, the exact reason for the low solubility of neat DBP into water is unknown. Overall, we rejected the hypothesis that DBP readily dissolves in water at a concentration of 100 μg/L. The results underlined the need first, for a solvent to deliver DBP to water, and second, for thorough mixing of the solvent in the water.

3.3 Study of the behaviour of DBP in a flow-through tank system

3.3.1 Introduction

Following the findings of the previous experiment, a tank system was set up in an attempt to track the movement of DBP in a flow-through system identical to that used in the pilot study in Chapter 2. The aim of this experiment was to measure the concentrations of DBP at various points in the experimental system, in order to gain a better understanding of whether or not:

i. DMF stock solutions were able to maintain DBP at their desired concentrations without degradation over time.
ii. DBP was consistently, homogeneously distributed in the tank water at near nominal concentrations.
iii. DBP was metabolised by the bacteria that accumulated in the tank.

3.3.2 Methods

As in Chapter 2, a peristaltic pump delivered a stock solution of DBP dissolved in DMF (5 g DBP/L) into a mixing vessel which mixed it with water from a header tank. Both the stock solution and mixing vessels were constantly stirred. Water from the mixing vessel flowed into a 38 L tank with an outflow at its opposite end (Figure 3.1). The flow rates of both water and stock solution were the same as used previously (500 ml water/min. mixed with 0.01 ml stock solution/min.). The nominal concentration was 100 μg DBP/L in all samples except the Blank samples. There were no fish in the tank. The experiment ran over 37 days from August 6th to September 10th, 2007, to allow bacterial growth to accumulate.

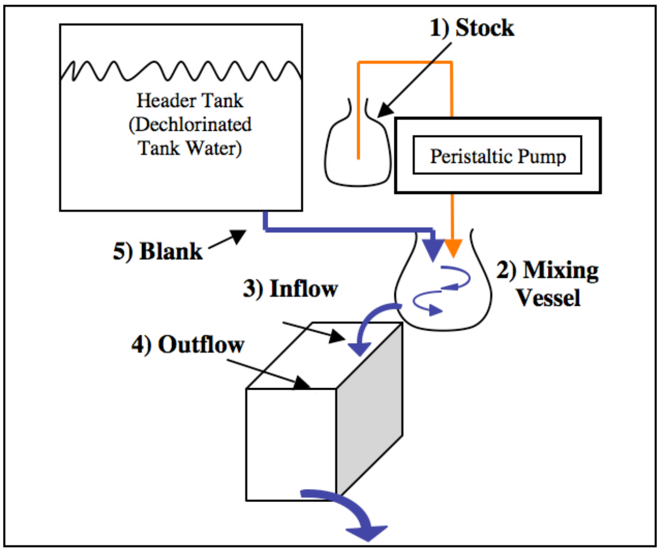


Figure 3.1. Depiction of the experimental flow through system and the various sampling points (1-5) used to track DBP for 37 days.

Water samples were collected from five different points in the system every 1-3 days for the first week, and weekly thereafter (Figure 3.1). On each sampling day, one 500 ml sample was taken from each sampling point for analysis. The Blank sample was taken from the outflow of the header tank. The Stock sample was prepared by collecting another 500 ml of water from the header tank, and spiking it with 10 μl of the stock solution for a nominal concentration of 100 μg DBP/L. The Mixing Vessel sample was taken from the outflow from the mixing vessel, while the Inflow and Outflow samples were taken by submerging the bottles in the tank water near the inflow and the outflow, respectively (Figure 3.1).

After water samples were collected, 5 ml of methanol were added to each one, and the contents mixed, to act as a preservative. The water samples were run through pre-conditioned Sep Pak C18 solid phase extraction cartridges (Waters Inc.) and analysed by GCMS with a standard repeated every 5 samples, as previously described (Chapter 2 Section 2.2.6.2).

One major assumption of this experiment was that solid-phase extraction was a reliable and accurate method for the extraction of DBP from water. Based on this assumption, water samples were not replicated, except for on Days 28 and 35. Six replicate samples were also collected from the mixing vessel on Day 37 (September 12th 2007), although no other water samples were collected on this day.

3.3.3 Results

The GCMS results were assembled from 3 different sample runs (August 23rd, and September 6th and 17th, 2007). The standard curves had very strong coefficients of determination on all days (R^2= 0.99). The coefficients of variation between the replicated standard samples during the GCMS runs varied from 19.0-47.3%. Generally, coefficients of variation up to 20% are considered to be acceptable in the quantification of chemicals by GCMS. Unfortunately, these samples were not rerun.

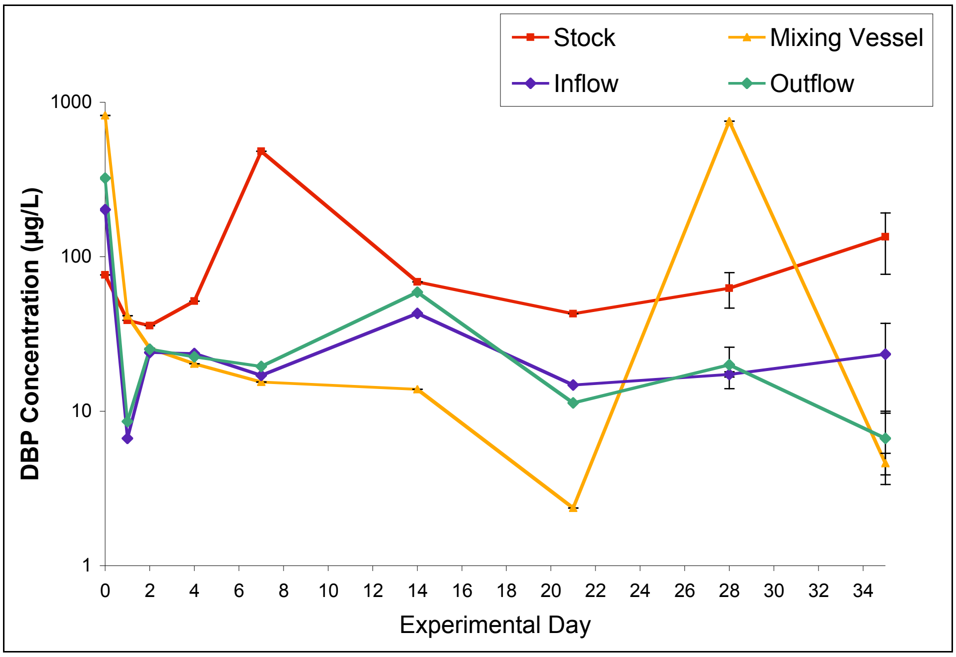


Figure 3.2. Concentrations of DBP in water on a logarithmic scale collected from various sampling points of a flow-through system over 35 days (mean ± SD for replicated samples).

Overall, the actual concentrations of DBP were much lower than the nominal concentration of 100 μg/L for the majority of the samples (Table 3.1). The Blank samples had little to no apparent DBP contamination with a mean concentration of 0.20 ± 0.11 μg DBP/L (SD) throughout the experiment. By contrast, at all other points, within 2 hours after having begun the peristaltic pump flow, the concentrations of DBP at all other points were much higher than expected. Subsequently, the concentrations were 2.4 to 59.0% the nominal concentrations at the mixing vessel, inflow, and outflow locations (with the exception of the Mixing Vessel sample on day 28) (Figure 3.2 and Table 3.1).

Table 3.1. Concentrations of DBP in water samples on each sampling day. The samples were collected from a flow-through system treated with a nominal concentration of 100 μg DBP/L for 37 days.

	DBP Concentrations in the Water (μg/L) on Each Sampling Day									
Sampling Point	Day 0	Day 1	Day 2	Day 4	Day 7	Day 14	Day 21	Day 28 (± SD)	Day 35 (± SD)	Day 37 (± SD)
Stock	76.0	38.8	35.7	51.7	480.4	68.6	42.82	62.6 ± 16.1	134.4 ± 57.3	-
Mixing Vessel	820.1	41.4	25.5	20.3	15.5	13.9	2.36	752.7	4.6 ± 0.7	40.2 ± 5.0
Inflow	201.3	6.7	24.0	23.6	17.0	43.0	14.81	17.3 ± 0.7	23.4 ± 13.7	-
Outflow	323.0	8.6	25.2	22.5	19.5	59.0	11.29	19.9 ± 6.0	6.7 ± 3.3	-
Blank	0.1	0.2	0.1	0.4	0.2	0.3	0.2	0.2	0	-

Contamination of the system with DBP or other phthalates did not appear to be a problem. The Blank samples contained measured concentrations of DBP ranging from 0 to 0.3 μg/L throughout the experiment. The concentrations of DBP in the spiked stock solution samples were fairly variable, but were generally the closest to the nominal concentration, with a mean of 110.12 ± 142.05 (± SD), while the median was 65.59 μg/L . The concentrations of DBP in the mixing vessel were also highly variable. For example, at times the DBP concentrations were only 2.4% of the nominal concentration, yet on Day 28 it was 7.5-fold higher than the nominal. With the exception of the samples collected on Day 0, the inflow and outflow generally yielded a much lower DBP concentration than expected, but were very similar on any given day (except Day 35). Over the course of the study their median concentrations were 20.36 (17.11, 23.53) for the inflow and 19.74 (13.34, 21.87) in the outflow, respectively (25th and 75th percentiles, respectively).

Due to the lack of replication, there is very little information on the degree of intrasample variation from Day 0 to 21. From the replicates collected on Days 28, 35, and 37, it appears that the sampling variation was relatively minimal sometimes, but did vary substantially at other times (see data from Day 35 in Table 3.1).

3.3.4 Discussion

The major shortcomings of this work were the lack of quality control measures. As a consequence, we were unable to assess the accuracy of any of the results. Despite this, the experiment did elucidate some of the behaviours of DBP in the flow-through system over time.

First, the high degree of variability in the concentrations in the stock and mixing vessel samples suggested that the DBP was not mixed homogeneously in the solvent or the mixing vessels. It was surprising that so much variation of DBP concentration was observed in the Stock samples, considering they were spiked with the same volume of stock each time. The very high DBP concentrations in the Stock samples on Days 7 and 35 suggest that the DBP may not have been properly dissolved in the DMF. Fortunately, they also suggest that DBP did not degrade in the stock solution over time. The inadequately mixed stock solution may also explain the large amount of variation in the mixing vessel. It is unlikely that the variation was due to changes in the flow rate of the DBP stock solution or the water, since these are known to be fairly stable. Several previously conducted experiments using this system have easily maintained concentrations of test chemicals of interest at concentrations close to nominal concentrations without difficulty (Giltrow *et al.* 2009; Harris *et al.* 2007). Another possible explanation for the variation in the concentration of DBP in the Mixing Vessels was the degradation of the solvent by bacteria. Increased microbial growth was first observed in the mixing vessel on Day 5. Perhaps the bacteria metabolised the DMF solvent, pulling the DBP out of solution. This idea is based on observations made on Day 35 of the experiment, when tiny globules of a clear oily substance were seen floating in the mixing vessel. It is likely these were globules of DBP. It is possible they had surfaced because the microbes were rapidly consuming the DMF, making the DBP insoluble in the water. These globules would introduce immediate variation in the mixing vessel samples, as the DBP would be concentrated at the surface of the mixing vessel, and hence could drastically alter its concentration in the tank itself. In addition to the variation, the bacteria may also be responsible for degrading the DBP itself, resulting in the low concentrations often measured in the Mixing Vessels, Inflow and Outflow samples.

Other conclusions we can draw from this experiment, are that the DBP was fairly uniformly distributed in the tank water, a conclusion supported by the similarity in the concentrations of the inflow and outflow samples over the experimental period. When multiple samples were collected from both the Inflow and Outflow points of the tank on Day 28, there was very little difference between their measured DBP concentrations, although this was not the case on Day 35. Thus, it appears that DBP can generally be homogeneously distributed throughout a tank and also maintained with relatively little variation over time.

3.3.5 Conclusions

The results from this study were complex. It appears that DBP did not degrade in the DMF. However, the amount of variation in the concentration of DBP in the Stock and Mixing Vessel samples was unexpected. The cause of this variation was unknown. It may have been due to poor dissolution in the DMF, from bacterial degradation of the solvent, or because the solid-phase extraction method was unreliable. At the time, we concluded that a different solvent for the dissolution of DBP into water might be more effective. However, it was unknown if this would improve the discrepancy between the measured and nominal concentrations of DBP in the water samples.

3.4 Experiment to test methanol as a more suitable carrier solvent for DBP

3.4.1 Introduction

Following the findings of the previous experiment, a more suitable solvent for the delivery of DBP to tank water was sought. Methanol was proposed as a potential candidate, as it is commonly used in fish exposure studies and is readily available. As with all solvents, methanol carries a risk of adversely affecting fish, but these risks were similar to risks associated with DMF exposure. Further, such risks are generally reported following exposure of fish to concentrations 5-fold higher than used in our system (Hutchinson *et al.* 2006). However, one study using methanol as a solvent observed the significant suppression of serum concentrations of 17β-oestradiol compared to a water control group (Harris *et al.* 2001).

The aim of this experiment was to determine two things:

i. If methanol is able to dissolve DBP in water more homogeneously than DMF.
ii. If the recovery of DBP from spiked water samples is adequate when compared to samples directly injected onto the GCMS.

We hypothesized that methanol would be able to more uniformly dissolve DBP into water than DMF, and that solid-phase extraction would recover >80% of the DBP from water.

3.4.2 Methods

On October 31st, 2007, two DBP stock solutions were prepared in either methanol or DMF at concentrations of 5 g DBP/L. For each solution, 2.5 g of DBP were weighed out in a glass weigh boat and dissolved in 500 ml of either solvent in a clean volumetric flask and mixed vigorously.

Two beakers containing 2 L each of clean water from the header tank were then spiked with 40 μl of one of the stocks to produce a nominal concentration of 100 μg DBP/L. Water from each beaker was then aliquoted into each of three 500 ml clean brown glass bottles. All six samples were extracted by solid-phase extraction and eluted following the same method as described previously (Chapter 2, Section 2.2.6.2).

Samples of each stock solution were also prepared to be injected directly onto the GCMS. Ten μl of each stock solution was added to a clean glass vial in triplicate, dried, and reconstituted in 1 ml of hexane and stored at 4°C to await GCMS analysis. We expected these samples would give an indication of how much DBP was present in the stock solutions that had not gone through the SPE process.

All samples were measured over two runs. Each sample was measured in triplicate on the GCMS (except for the third DMF extraction). The samples spiked with DMF were analysed November 1st, 2007, at which time the GCMS ended abruptly due to a programming error. Thus there was no replication of standards in this case. The methanol extractions and all direct injection samples, however, were run without incident on November 12th, 2007, with a standard repeated every 5 samples.

3.4.3 Results

Results from the GCMS runs reported a lower-than-desired precision, with the coefficient of variations ranging from 35-70% between repeated standards. However, accuracy for both runs was indicated by large correlation coefficients (R^2=0.99). Two sample replicates were omitted from the GCMS results: one reading from the first methanol direct injection sample, and another from the second methanol direct injection sample. These samples were injected into the GCMS three times with two very similar readings. In both cases, the third reading was omitted on the grounds that it was more than two standard deviations lower than the mean.

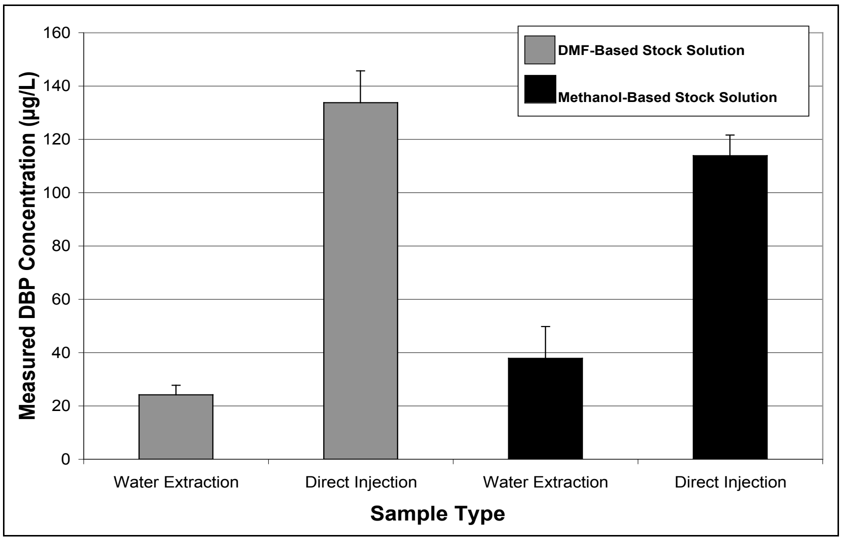


Figure 3.3. DBP concentrations (mean ± SEM) measured in water samples extracted by SPE, or in samples directly injected directly onto the GCMS. The nominal concentration for all samples was 100 μg DBP/L.

Both the DMF and methanol stocks were able to dissolve DBP into water samples consistently, although the concentrations of DBP in the SPE-extracted samples were much lower than expected. The samples using DMF as a solvent were measured to have only about 22% of the nominal concentration of DBP, whereas the water samples spiked with DBP in methanol appeared to have higher concentrations: about 38% of the nominal concentration. In contrast, the concentrations of DBP in the samples injected directly onto the GCMS was similar whether DMF or methanol was used; both exceeded 100 μg/L (Figure 3.3).

3.4.4 Discussion and Conclusions

This experiment revealed some quite surprising and important results despite the high coefficients of variance reported by the GCMS. The high DBP concentrations in the directly injected stock solutions indicated that the DBP had not been degraded or “lost” between its dissolution into a solvent and its analysis. The fact that these concentrations exceeded the nominal concentration was more likely a result of the inherent inaccuracy in the analytical method. In other words, the preparation of the diluted standards, and/or the variation on the GCMS, are probably the reason the concentrations in the direct injection exceeded 100 μg DBP/L. After all, the concentrations reported by the GCMS are only relative to the nominal concentration of the standards. Overall, these results did not discount the ability of DMF to act as an appropriate solvent, and they also supported the hypothesis that methanol can dissolve DBP in a homogeneous solution.

Considering the disparity between DBP concentrations in the directly injected samples versus the water samples extracted by SPE, it appears that either the DBP adsorbed to the glassware upon addition to the water, or that solid-phase extraction methods were inappropriate in the analysis of DBP.

In conclusion, firstly, due to the problems encountered with DMF, and despite any clear differences between the two solvents, methanol was chosen as the new solvent for DBP. Secondly, solid-phase extracted samples were shown to recover significantly less DBP than was present. Therefore, a new method for the extraction of DBP from water needed to be established.

3.5 Development of a new method for the extraction of DBP from water

3.5.1 Introduction

Liquid-liquid extraction (LLE) was suggested as an alternative method for the extraction of DBP from water samples (personal communication, Dr. Mark

Scrimshaw at the Institute for the Environment). The principle of LLE is similar to reverse-phase SPE, in that when a lipophilic solute dissolved in water comes into contact with a more lipophilic phase (octadecylsilane in SPE, and an organic solvent in LLE), it is theoretically drawn out of the water and onto/into the other, lipophilic phase where it is more "comfortable". In the case of LLE, it can be said that like dissolves like. Thus DBP, a non-polar liquid, will move from the water sample and dissolve in the organic solvent. This phase, containing the DBP, is then collected and the solvent is evaporated off, leaving the extracted DBP, which is reconstituted in a known volume of hexane for injection into the GCMS.

We designed an experiment to assess the accuracy and reliability of LLE for the extraction of DBP from water and hypothesized that it would be a more accurate and reliable method than solid-phase extraction.

3.5.2 Methods

A stock solution of 0.5 g DBP/L was prepared in methanol. Two hundred microlitres of stock was then added to 1 L of water in a clean glass volumetric flask, for a final concentration 100 μg/L, and mixed well with a stir bar (10-15 minutes). Three 250 ml aliquots were then decanted into volumetric flasks, which were liquid-liquid extracted.

The LLE involved first adding 5 ml of hexane (HPLC grade, Rathburn Co.) to each water sample with a glass pipette. Each sample was shaken vigorously for approximately 20 seconds, every 5 minutes, for 30 minutes. After this period, the samples were left to settle until hexane bubbles could no longer be seen floating to the surface (this took approximately 5 min.). The hexane was drawn off with a clean glass pipette and placed in a clean glass vial. The hexane samples were dried under a stream of nitrogen at room temperature for 1-3 hours, depending on the amount of residual water present. Once dried, samples were reconstituted with 1 ml hexane, wrapped in parafilm, and stored at 4°C to await analysis (Figure 3.4). The expected concentration in each aliquot measured on the GCMS was 25 mg/L.

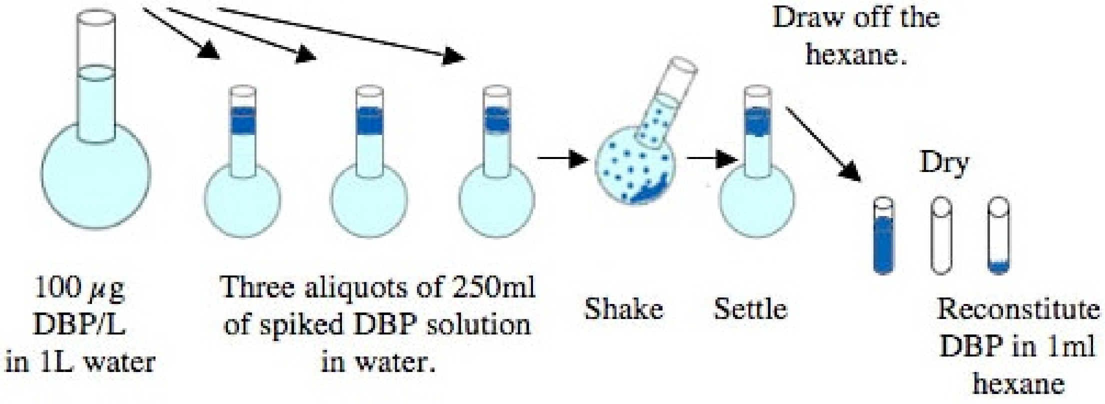


Figure 3.4. Depiction of the liquid-liquid extraction method, using hexane (dark blue), to extract DBP from spiked, aliquoted water samples (light blue).

Direct Injection samples were also prepared in triplicate by adding 50 µl of stock solution to a clean elution vial, drying under a stream of nitrogen (10 minutes) and reconstituting with 1 ml hexane for a target concentration of 25 mg/L. Samples were analyzed on the GCMS on November 30th, 2007, with a standard repeated every 5 samples.

3.5.3 Results

The GCMS run appeared to be successful, with coefficients of variation for the repeated standards of less than 3.6%. The correlation coefficient for the standard curve was also very strong (R^2=0.99).

The recovery of DBP from the direct injection samples was consistent at 17.74 ± 1.02 mg/L (mean ± SD), but it was lower than the expected concentration of

25 mg/L. However, the liquid-liquid extracted samples recovered almost 100% of what was measured in the direct injection samples (17.52 ± 0.36 mg/L (mean ± SD)) (Figure 3.5).

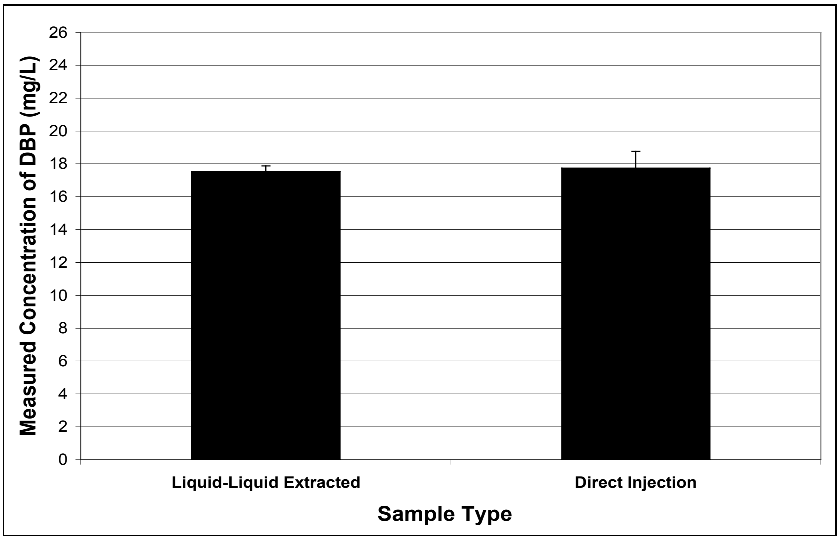


Figure 3.5. Measured concentrations (mean ± SD) of DBP in liquid-liquid extracted water sample compared to directly injected stock samples.

3.5.4 Discussion & Conclusions

The liquid-liquid extraction method used in this experiment appeared to efficiently extract the DBP from the water samples (Figure 3.5). Recovery of the phthalate in this experiment was much higher than was achieved using solid-phase extraction (Figure 3.3). This result provides ample support for the hypothesis that liquid-liquid extraction is a more appropriate method for the measurement of aqueous concentrations of DBP.

This finding also put into question the accuracy of the concentrations measured in the pilot study involving exposing fathead minnows in Chapter 2. It suggests that the measured concentrations of DBP reported in Chapter 2 were not representative of the actual concentrations of phthalate in the fish tanks, because of the use of the SPE method. Thus, it is likely the fathead minnows in both the F_0 and F_1 generations were exposed to DBP concentrations much higher than those reported, and nearer the target (nominal) concentration.

In conclusion, the liquid-liquid extraction method developed here was chosen to replace solid-phase extraction in subsequent experiments where phthalate concentrations needed to be measured. Further, the use of replicated samples, and samples of stock to be directly injected, were also incorporated into the protocol to further improve the precision and accuracy of the technique.

3.6 A study of the uptake of DBP by the fathead minnow: Part one

3.6.1 Introduction

Following the establishment of a reliable method for the extraction and analysis of DBP in water, it seemed necessary to provide some evidence that not only can DBP dissolve in water, but that it is taken up by fish. I aimed to demonstrate the uptake and bioconcentration of DBP by fish indirectly, by measuring a decrease in the DBP concentration in the water after fish had been added to a tank.

The theory behind this indirect approach was mainly based on the known ability of fish to rapidly absorb lipophilic chemicals via the gills (Randall *et al.* 1996). Further, similar work conducted in juvenile and adult rainbow trout (*Salmo gairdneri*), and in the sheepshead minnow (*Cyprinodon varieagatus*) showed that increases in the tissue concentrations of radiolabelled DEHP could be measured by a parallel decrease of radiolabelled DEHP in the tank water (Karara and Hayton 1984; Tarr *et al*. 1990).

We hypothesized that if fish were added to a flow-through system treated with DBP, they would be able to absorb it at a rate fast enough to cause a measurable decrease in the DBP concentration in the water.

3.6.2 Methods

This experiment involved the use of three tanks, one Solvent Control and two DBP treatment tanks at nominal concentrations of 10 and 100 μg DBP/L. Stock solutions were prepared in methanol, and the flow through system was set up as described in Chapter 2 (Section 2.2.2).

The experiment was run for 24 days (January 14th and February 24th, 2008). Twelve adult fathead minnows (6 males and 6 females) were added to each tank on Day 10 (at 240 h). Water samples prior to the addition of the fish were collected on days 2, 3, 4, and 7. Water samples were collected just prior to their addition, and then 1, 3, 6, 12, and 18 hours afterwards. Additional samples were collected on Days 11, 12, 13, 14, 15, 16, 17, 19, 21, and 24 (highest concentration tank only on this day).

Samples were collected in duplicate from the DBP-treated tanks both from the centre of the tank itself and from the inflow (the outflow of the mixing vessel). Solvent Control samples were not replicated, since previous work suggested contamination of the tank water by phthalates was not of particular concern. Sample volumes were 1 L for each Solvent Control and 10 μg DBP/L sample collected, and 250 ml for those from the 100 μg/L DBP tank. The water samples from the 10 and 100 μg DBP/L tanks not were collected in duplicate on Days 2, 3, 10 (241 hours), and 17, due to time constraints.

The samples were liquid-liquid extracted within 3 hours of collection, except for samples taken on Day 10 (at 252 hours), and Days 12 and 19, which were left overnight and extracted after 12-18 hours. A 1:50 hexane to water volume ratio was used to conduct the liquid-liquid extractions. The 250 ml water samples were shaken with hexane for 15 min., and the 1 L samples for 30 min.. All LLE samples were then treated as described in Section 3.5.2.

Initially, 1 L of water was spiked with stock and extracted alongside tank samples as a standard. Additionally, 100 μl of an internal standard of DEP (2.5 g/L in methanol) was also added to each sample. This was to help to determine the amount of recovery of DBP from each individual water sample. However, DEP was inadequate as an internal standard due to its lower affinity for the organic phase ($\log K_{ow}$ of 2.38). Thus, on Day 8, 100 μl of benzyl butyl phthalate internal standard (2.5 g BBP/L methanol) was instead, added to each water sample prior to extraction. BBP was a more appropriate internal standard because its $\log K_{ow}$ is very similar to that of DBP (4.59 and 4.45, respectively) (Staples *et al.* 1997). Thus, their affinities for the organic solvent were found to be more similar. The BBP internal standard was stored in a dark glass vial at 4°C. The expected concentration of the internal standard in each sample was 25 mg/L, which was verified by direct injection.

Direct injection samples were prepared on January 22nd 2008. The internal standard samples were prepared by pipetting 100 μl of the BBP internal standard into a clean vial, evaporating the hexane under a stream of nitrogen gas, and reconstituting them with 1 ml hexane. Both Solvent Control, 10 and 100 μg DBP/L stock solutions were also prepared as direct injection samples in triplicate. The Solvent Control and 10 μg DBP/L direct injection samples were prepared by adding 20 μl stock to the vials, evaporating, and reconstituting them in 1ml of hexane. The

100 μg DBP/L tank direct injection samples were prepared by adding 10 μl of the stock solution to a vial, drying, and reconstituting it with 2 ml hexane. All samples were wrapped in parafilm and stored at 4°C until analysis.

Due to problems, the GMCS analysis was conducted for the DBP-treated tanks over several runs, on February 3rd, 5th, 6th, 15th and 22nd 2008. The Solvent Control tank samples were run April 12th, 2008. A standard was repeated every 5 samples for all runs. The concentrations of DBP in the water samples were calculated based on the percent recovery of BBP from each sample. One hundred percent was determined as the concentration of BBP measured from the directly injected internal standard samples on the GCMS in each run. DBP concentrations in the samples were not calculated based on the internal standard recovery if it exceeded 100%, since it was assumed that >100% recovery of BBP indicated that 100% of the DBP had been extracted. Where no BBP standard was used (eg. Days 2-8 and some other samples not spiked due to technician error), the concentrations are reported as measured, and are indicated in Table 3.2.

3.6.3 Results

Overall, the GCMS runs reported an acceptable level of intrasample variation with coefficients of variation mainly within 20%. On a few occasions, the coefficients of variation for some concentrations of the standards reached up to 32%. However, the accuracy of all GCMS runs was fairly high, with coefficients of determination above R^2=0.95.

The measured concentrations of the samples from the inflow were highly variable compared to those of the tank samples (as observed previously in Section 3.3.3). Thus, only the DBP concentrations from the samples collected from the tank itself are presented. The BBP internal standard was measured with 100% recovery measured at 15.03 mg/L on the GCMS. This was not as high as expected (25 mg/L). The recovery of the internal standard from the extracted samples was 70.44 ± 6.10% and 60.99 ± 1.79% from the 10 and 100 μg/L tanks, respectively (mean ± SD).

Table 3.2. DBP concentrations (mean ± SD) calculated based on the percent recovery of the BBP internal standard in three tanks: Solvent control, 10, and 100 μg DBP/L (n=2, except where indicated).

Sample Time Days (Hours)	DBP Concentration in the Water Sample (µg/L)						
	Solvent Control Tank	Low Dose Tank (10 µg DBP/L)			High Dose Tank (100 µg DBP/L)		
		Mean	±	SD	Mean	±	SD
2 (48 h)	0.00	0.00	±	0.00[a, c]	84.84	±	0.00[a, c]
3 (72 h)	0.00	8.85	±	0.00[a, c]	76.08	±	0.00[a, c]
4 (96 h)	0.00	13.94	±	1.32[c]	80.56	±	2.45[c]
7 (168 h)	0.00	8.77	±	1.03[c]	67.40	±	5.06[c]
8 (192 h)	0.00	10.07	±	0.00[a]	75.67	±	5.12
9 (216 h)	-		-		79.55	±	6.30
9.5 (222 h)	0.00	7.45	±	0.00	75.60	±	0.00
10 (240 h)	0.00	4.66	±	0.00	60.21	±	6.92[a]
- - - - - - - - - - - - - Fish addition - - - - - - - - - - - - -							
10 + 1h (241 h)	0.00	5.19	±	0.00[a]	39.26	±	0.00
10 + 3h (243 h)	0.00	5.72	±	0.76	48.17	±	2.31
10 + 6h (246 h)	0.00	6.39	±	0.93	54.19	±	3.05
10 + 12h (252 h)	0.00	3.04	±	1.58[b]	31.81	±	5.45[b]
11 (264 h)	0.66	5.66	±	0.73	55.80	±	7.75
12 (288 h)	0.00	1.11	±	1.08[b]	26.27	±	3.45[b]
13 (312 h)	0.00	5.66	±	0.73	47.44	±	0.00[a]
14 (336 h)	0.00	5.18	±	0.35	48.22	±	2.62
15 (360 h)	0.00	6.82	±	1.62	43.97	±	1.10
16 (384 h)	0.00	6.23	±	0.55	48.24	±	2.63
17 (408 h)	0.00	8.52	±	0.00	68.71	±	0.00[a]
19 (456 h)	0.00	3.57	±	0.05[b]	16.60	±	1.34[b]
21 (504 h)	0.00	7.87	±	0.54	66.26	±	3.47
24 (576 h)	-		-		61.64	±	6.55

[a] No duplicate sample was collected

[b] These samples were collected approximately 12-18 hours prior to extraction.

[c] No BBP internal standard was used; concentrations are reported as measured.

Some very low concentrations were measured from samples collected on Day 10 + 12 hours (252 h), and Days 12 and 19 in both the 10 and 100 μg/L tanks. These samples were those that had been collected more than 12 hours prior to their extraction (Table 3.2). The parallel drops in DBP concentrations in these sample in both tanks can be seen in Figure 3.6. It suggests the DBP degraded in the water samples before they were extracted, and thus these data were omitted from discussion of the results (Figures 3.7 and 3.8).

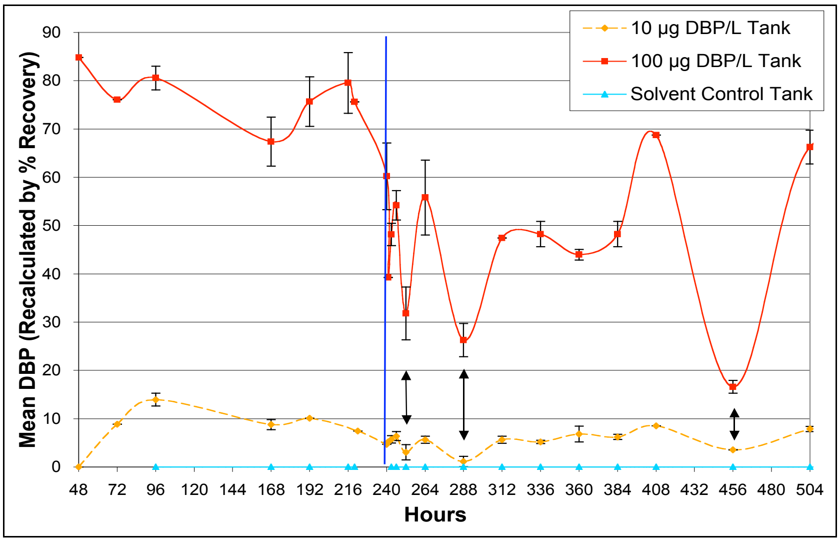


Figure 3.6. Concentrations of DBP (mean ± SD) in Solvent Control, 10, and 100 μg DBP/L tanks. Concentrations are calculated according to the percent recovery of internal standard in each sample. Fish were added at 240 hours (blue vertical line), and samples that were extracted >12 hours after collection are indicated by arrows.

The Solvent Control tank was generally free of any phthalate contamination. However, DBP was detected in the inflow to the Solvent Control tank (but not the tank water samples) on Days 10, 11 and 17 at concentrations of 1.84, 0.66 and 1.07 μg DBP/L, respectively.

The concentration of DBP in the 10 μg DBP/L tank was fairly variable over the course of the experiment (Figure 3.7). The DBP concentration ranged from 0 to 13.6 μg DBP/L, with a mean (±SD) of 6.24 ± 3.12 μg DBP/L. Before the addition of fish at 240 hours, the concentration of DBP had been in decline from 13.94 ± 1.32 μg DBP/L at 72 hours, to 4.66 μg DBP/L by 240 hours. There was no evidence of a decline in DBP concentration following the addition of the fish. After they had been added, there was actually an increase in the DBP tank water concentration, although this was relatively small (an increase of 1.7 μg DBP/L between 241 and 246 hours). One week after the addition of the fish, the concentration increased, reaching a maximum concentration of 8.25 ± 0.17 μg DBP/L on Day 17 (408 h).

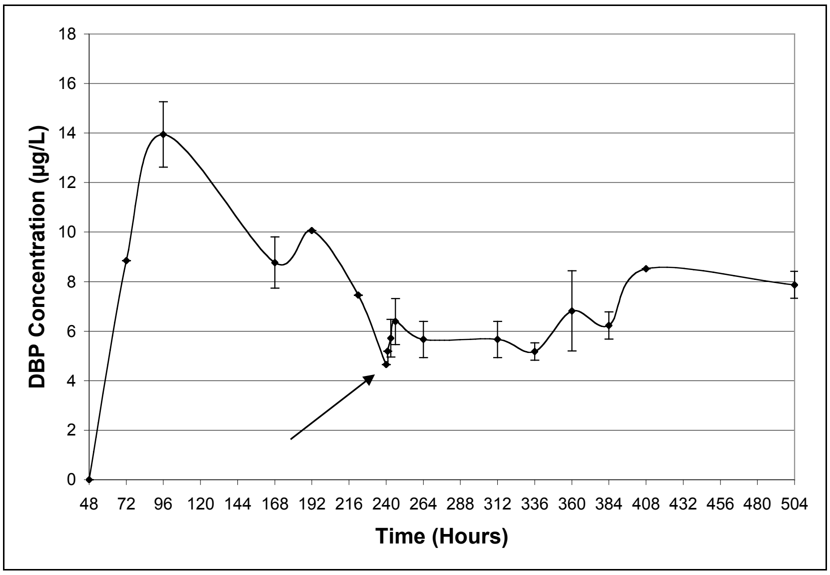


Figure 3.7. DBP concentrations (mean ± SD) in the 10 μg DBP/L tank, calculated based on the recovery of BBP from each sample. Data from the samples that were not extracted within three hours of collection were omitted. (The arrow indicates the sample collected just prior to the addition of the fish).

The concentration of DBP in the 100 μg/L treatment tank was slightly more stable (Figure 3.8). The mean (±SD) concentration of DBP in the tank water throughout the experiment was 62.17 ± 14.07 μg/L, although this was only 62% of the nominal concentration. In the first 10 days leading up to the fish addition, the concentration of DBP was maintained at or above 58 μg/L. Just before the fish were added, the concentration of DBP in the water samples collected at 240 hours was 58.17 ± 17.34 μg DBP/L (mean ± SD). After the fish were added, the DBP concentration immediately decreased, reaching the lowest concentration measured, 39.26 μg DBP/L, over the entire experiment just 1 hour later. The DBP concentration appeared to take 6 hours to increase after the addition of the fish, reaching 54.19 ± 3.05 μg/L (Day 10, 246 h) (Figure 3.8).

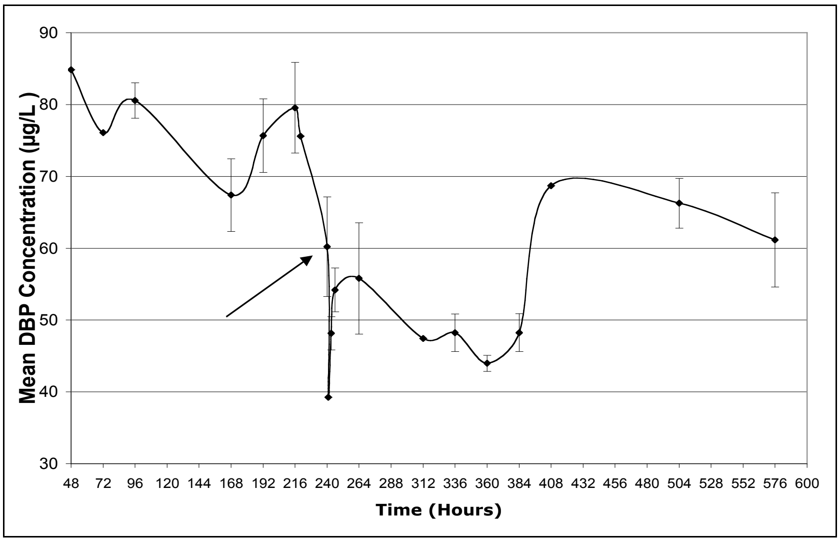


Figure 3.8 Concentrations of DBP (mean ± SD) from the 100 μg/L tank, calculated based on the recovery of BBP from each sample. Data from samples that were not extracted within 3 hours after collection were omitted. The arrow indicates the sample collected just prior to the addition of the fish.

3.6.4 Discussion & Conclusion

This experiment suggested that a decline in the concentration of DBP in a flow-through system can be observed following the addition of fish. While this was only apparent in the 100 μg DBP/L tank, it appears to suggest that fish can rapidly bioconcentrate aqueous DBP. However, it was also apparent that within a few hours after the addition of fish to the tanks, the concentration of DBP returned to levels similar to those observed prior to their addition. Hence, it is unlikely that the lack of

DBP measured in the water samples collected in the pilot study (Chapter 2) was a result of the complete uptake of DBP by the fish in these tanks.

The reason that a similar effect was not observed in the 10 μg DBP/L tank is probably due to the fact that it was very difficult to maintain a consistent concentration of DBP at 10 μg/L. Any reduction of DBP caused by absorption into fish tissues was likely obscured by the large amount of variation in the concentration of DBP in the tanks over time.

An interesting discovery during this experiment was the finding that water samples collected approximately 12-18 hours prior to their extraction had substantially lower concentrations of DBP than samples extracted within three hours of collection (Figure 3.6). This provided some indication that DBP is not particularly stable in water, and may help to explain why the initial samples collected in the pilot study (Chapter 2) were found to contain concentrations of DBP much lower than expected. It may also explain why the samples sent for analysis by an external agency early on in the pilot study were found to have no measurable concentrations of DBP.

One problem consistently encountered was the low recovery of the BBP internal standard from some of the water samples. This may be because, by Day 7 of the experiment, bacterial growth had begun to accumulate. The bacteria facilitated the emulsification of hexane during LLE and made recovery of the hexane from the water samples very difficult. This was somewhat circumvented by the use of an internal standard to calculate recovery, but was not ideal. Unfortunately, this led to recoveries of the internal standard as low as 17.5%. It should be noted that little-to-no phthalate contamination was observed in the Solvent Control, even when there was 100% recovery of the internal standard. This suggests that the zero-values for the concentrations of phthalates in the Solvent Controls were not false negatives. Regardless, the lower the recovery of DBP and BBP from water samples, the less accurate recalculation of the DBP concentrations became, and therefore it seemed that a more reliable method for extraction was required.

In general, this experiment provided insight into some areas for improvement:

i. Several direct injection samples of the internal standard should be prepared and run alongside LLE samples in each run, so that the maximum amount of recovery of BBP can be determined more accurately.

ii. The LLE method developed requires further changes to minimize emulsification and increase the recovery of both DBP and the internal standard.

iii. A repetition of this experiment would be useful to increase the weight of evidence demonstrating the ability of fish to measurably reduce the concentration of DBP in water.

3.7 Optimization of the liquid-liquid extraction method

3.7.1 Introduction

In response to the results of the previous experiment, some suggestions were made to improve the recovery of phthalate from water using the liquid-liquid extraction method (Dr. Mark Scrimshaw, personal communication). Suggestions included:

1. Glass separation funnels be used instead of volumetric flasks.

2. A high-density solvent such as dichloromethane (DCM) (HPLC grade, Fisher Scientific Ltd.) be used instead of the low-density hexane for LLE.
3. A salt be added to the water sample during LLE to mitigate emulsification of the water with the organic phase.

3.7.2 Methods

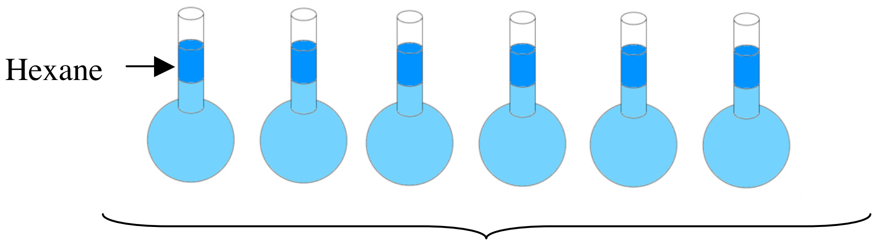
To test this amended method, an experiment was conducted on February 27th, 2008. Six litres of water from the Solvent Control tank, which had been maintained since the previous experiment, was collected in a glass beaker. This water contained a fair amount of bacterial growth, and thus would present the worst case scenario to test the method. One hundred and twenty microlitres of DBP stock (5 g/L in methanol) was added to the water to give a nominal concentration of 100 μg/L, and mixed well. A clean volumetric flask was then used to aliquot 250 ml of the water into each of six separation funnels (for extraction with DCM) and six volumetric flasks (for extraction with hexane). Each aliquot was then spiked with 100 μl of BBP as the internal standard (0.25 g/L in methanol), for the same nominal concentration in each sample as before, namely 25 mg/L. Each aliquot was stored on ice prior to extraction. The six volumetric flasks were extracted with 5 ml hexane according to the methods described in Section 3.6 for 250 ml volumes; however, each extraction was repeated.

The water samples in the separation funnels were extracted with DCM. Three aliquots were shaken twice with 10 ml of DCM for 5 minutes each. The remaining three aliquots were shaken twice with 10 ml DCM (3 minutes) and once with 5 ml DCM (1 minute) (Figure 3.9). Potassium chloride salt (Sigma Aldrich

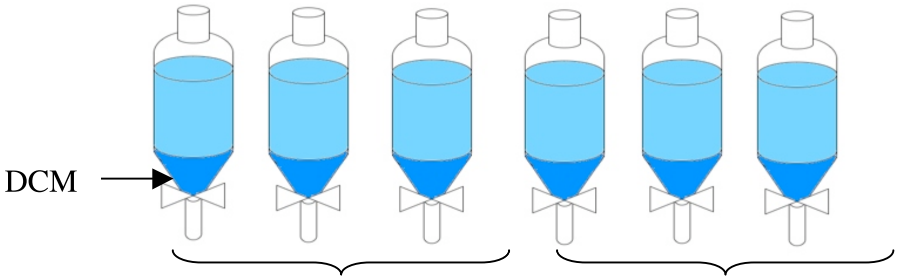
Ltd.) dissolved in clean de-ionized water (Milli-Q) was added to all water samples (both hexane and DCM extractions) prior to the collection of the final extract to help saturate out any of the organic phase and maximise recovery.

All extracts were collected in clean vials, dried under a stream of nitrogen at room temperature, and reconstituted in 1 ml hexane. Samples were sealed with parafilm and stored in the refrigerator at 4°C until analysis.

Three replicated samples of stock solutions were prepared for direct injection onto the GCMS (at a concentration of 25 mg/L of both DBP and BBP) by adding 10 μl of DBP stock and 200 μl BBP stock to a clean vial, drying with nitrogen gas, and adding 2 ml hexane. The GCMS run for all samples was conducted on March 1st, 2008, with a repeated standard sample included after every fifth sample.



(a) Six volumetric flasks each extracted twice with hexane.



(b) Three separation funnels extracted twice with dichloromethane.

(c) Three separation funnels extracted three times with dichloromethane.

Figure 3.9. Depiction of the two liquid-liquid extraction methods that were compared.

3.7.3 Results

The variation in the measured concentration of the standards from the GCMS was quite small, with coefficients of variation below 20% between repeated standards. The standard curves for both DBP and BBP also suggested a high degree of accuracy ($R^2 = 0.99$ in both cases).

One hundred percent BBP was assigned as the mean (±SD) amount of BBP measured from the 3 directly injected samples (14.63 ± 0.98 mg/L). The recovery of BBP for all of the extracted samples was 109.6 ± 11.98% (mean ± SD).

Overall, the hexane method of extraction appeared to have a similar degree of recovery, although with slightly more variability than the method using DCM. In terms of the DCM LLE, three repeated extractions proved to be more efficient than two (with recoveries of 120 ± 3.41% and 99 ± 4.26%, respectively). With recovery of the BBP internal standard often exceeding 100%, it is likely that the concentrations of BBP in the direct injection samples were lower than usual. Thus, the measured concentrations of DBP are presented as measured by the GCMS, without calculation based on percent recovery of the internal BBP standard. The mean DBP concentration of the 2 x DCM extractions was 79.97 ± 1.19 μg/L, and was 87.68 ± 1.03 μg DBP/L (mean ± SD) for the 3x DCM extractions (Figure 3.10). Overall, there was a good recovery of DBP from water samples using both the old and new LLE methods, with variation between samples at an acceptable level (within 20%).

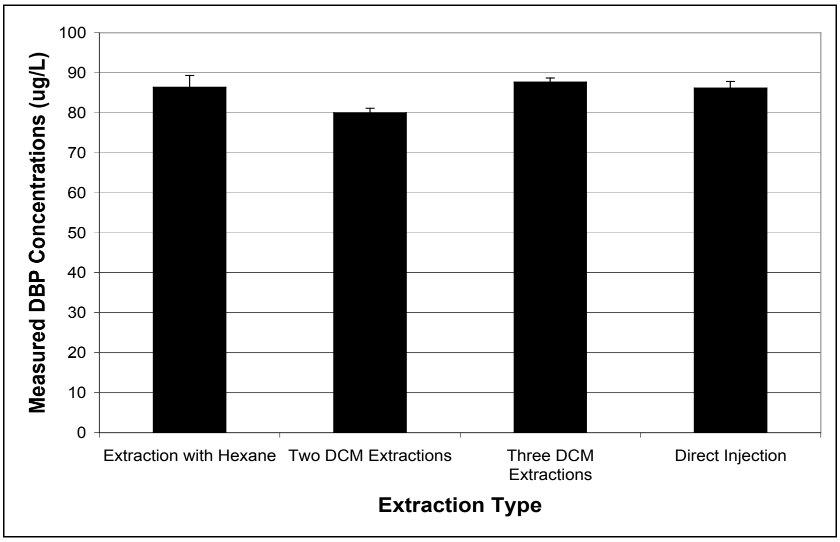


Figure 3.10. Measured concentrations of DBP in the extracted water samples (mean ± SD). The concentrations were not calculated based on percent recovery of the BBP internal standard.

3.7.4 Discussion & Conclusions

The LLE method with DCM was easier to conduct than the method using hexane. This was mainly because of the ease of drawing the denser DCM off from the bottom of the separation funnel, which helped to increase the recovery of the organic phase, and thus of the DBP. However, this ease was not reflected in the results (Figure 3.10). The use of replicated samples and "direct injection" samples were also very useful in improving the reliability of the technique.

The BBP internal standard calculation was clearly open to some problems due to recovery exceeding 100% for many extracted samples. Thus, this method is not completely accurate, but the internal standard does provide a useful quality control indicator for the extraction method.

In conclusion, we decided to use the optimized liquid-liquid extraction method involving the use of DCM in separation funnels with a salt solution added at the end, the BBP internal standards, and both DBP and BBP direct injection samples for the extraction of DBP from water samples in any subsequent work.

3.8 A study of fish uptake of DBP by the fathead minnow: Part two

3.8.1 Introduction

Once an optimized method for the extraction and measurement of DBP in water was established, we decided to repeat the experiment conducted in January, 2008, to provide further support for the hypothesis that the uptake of DBP by fish could be measured indirectly via the reduction in the concentration of DBP in the water.

3.8.2 Methods

The design of this experiment was much simpler than the one previously conducted. Only one tank was set up with a nominal concentration of 100 μg DBP/L for seven days (March 7th to 14th 2008). The DBP stocks were delivered to the tank in a manner identical to that described in Section 3.6. Prior to the beginning of the experiment (at time 0), the stock solution and water were pumped into the tank for approximately four hours in order to allow the stock flow rate to stabilise to 0.01 ml/min. Once stable, the experiment was initiated, and the first water samples were collected.

Water samples were collected daily from the tank itself. Six male and six female fathead minnows were added on Day 3, just after the 72-hour water samples were collected. Following their addition, water samples were collected after 15 min., 30 min., 1, 1.5, 2, 2.5, 3, 6, and 12 hours, and daily until Day 7 (that is, four days after the introduction of the fish).

All water samples were collected in duplicate in 250 ml volumetric flasks by submerging them in the tank water, and were extracted within 3 hours of collection. Prior to extraction, each sample was decanted into separation funnels and 100 μl of a BBP internal standard (0.25 g/L in methanol) was added. The extraction of each water sample involved 3 extractions with DCM: two of 10 ml, shaken for 3 minutes each, and one of 5 ml shaken for 1 minute. Potassium chloride salt was added to the water samples prior to the collection of the last DCM phase, as described in Section 3.7. This salt helped to precipitate out any emulsified DCM in the water phase, ensuring the maximum amount of DCM was recovered. DCM was collected in clean 25 ml vials, dried, and stored as described previously (see Section 3.7.2).

Direct injection samples of BBP internal standard (at 25 mg/L) were prepared as previously described. In brief, on each sampling day 100 μl of BBP standard stock and 10 μl DBP stock were added to a clean vial, which was dried, reconstituted with 1 ml hexane, wrapped in parafilm, and stored at 4°C until analysis.

Due to problems encountered with the GCMS, the samples were not analysed in one complete run. The samples were analysed in their entirety by GCMS first on April 1st and April 7th, 2008. All of the samples were then repeated in another two consecutive GCMS runs on on April 8th and 9th 2008 in hopes to improve accuracy. It should also be noted that one of each of the duplicated samples collected at 73.5 and 74 hours were extracted incorrectly and therefore not run on the GCMS.

3.8.3 Results

The GCMS run on April 7th was well within the 20 percent variation for both DBP and BBP standards. However, the run on April 1st was not as precise as desired with a coefficient of variation of in the BBP standards of up to 42%. By contrast, the consecutive runs on April 8th and 9th were less variable, with coefficients of variation <16%, except for the 2.5 mg/L standard on April 8th (23%). All standard curves on all days had coefficients of determination exceeding $R^2=0.92$. Due to the lower coefficients of variation, we will discuss the results from the runs on April 8th and 9th 2008 only. However, the general trends in DBP concentration were very similar between the earlier and later GCMS runs. Further, all GCMS analyses reported a mean (± SD) internal standard recovery of 95.88 ± 2.61%.

There was a trend of increasing DBP concentration until 72 hours (Day 3), just prior to the fish addition. Over the first three days of the experiment, the concentration increased from 80.06 ± 1.80 to 157.06 ± 18.12 μg/L (mean ± SD). After the fish were introduced, the concentration of DBP decreased by approximately 51 μg/L over the following hour to 106.81 ± 2.66 μg/L . The concentration of DBP appeared to then stabilise at 74 hours (2 hours after the fish had been added) (Figure 3.11).

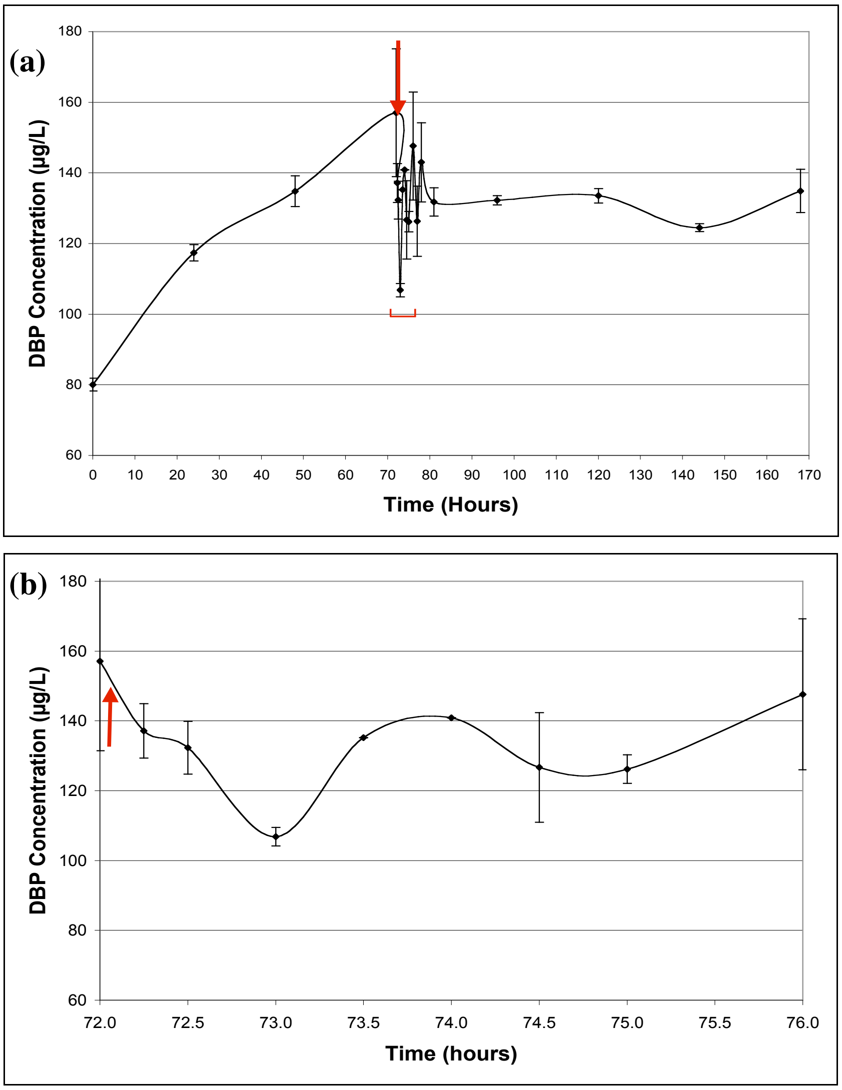


Figure 3.11. Concentrations of DBP (μg/L) (mean ± SD) in water samples collected from a tank containing a nominal concentration of 100 μg DBP/L for 7 days. (a) The concentrations of DBP over the entire 7 days. The brackets indicate the enlarged area shown in (b). (b) A detail of the concentrations of DBP in the tank four hours after the addition of the fish. (Concentrations are calculated based on the percent recovery of BBP internal standard in each sample. The arrow indicates when the fish were added to the tank).

3.8.4 Conclusions

The data presented in this experiment provide further support for the hypothesis that the uptake of DBP by fish can be measured indirectly via water chemistry. The addition of fish was followed by a decrease of 51 μg DBP/L after one hour. It is highly possible that this decrease was caused by the uptake of DBP into the fish tissues via the gills, as DBP is a small lipophilic substance easily absorbed by fish (Staples *et al.* 1997). However, it is also possible that the decline in DBP concentration following the fish addition was simply due to variation of the DBP concentration in the tank. Initially the DBP concentration did vary by as much as 32 μg DBP/L over the first three days of the experiment on its own. However, the concentration of DBP one hour after the addition of the fish was the lowest measured DBP concentration over the entire course of the experiment. Further, the similar pattern of DBP concentration between the repeated experiments provides a strong weight of evidence that fathead minnows bioconcentrate DBP to the extent that it can be observed within one hour as a decrease in the concentration of DBP in the tank water.

3.9 General Discussion & Conclusions

Overall, the experiments in this chapter provided the basis for further investigation of the endocrine disrupting properties of DBP in fish. It is essential that during exposures of fish to environmentally relevant concentrations of pollutants:

i. The chemical concentrations are monitored.
ii. There is sufficient evidence that the chemical is taken up by the fish, where it is able to elicit biological effects.

The work discussed in this chapter succeeded in establishing sound and reliable methods for the analysis of DBP concentrations in water. These results were also essential in confirming that the flow-through system used to expose fish to DBP, succeeded in doing so in an accurate and consistent manner. In other words, I confirmed that:

i. DBP concentrations were maintained in the tank water within an acceptable range (ie. they were relatively similar to nominal concentrations).

ii. The various DBP concentrations could be maintained in a gradient, so that different treatment groups were distinct from one another.

iii. Contamination by DBP, as well as other phthalates, was not a problem.

iv. Fish exposed to aqueous concentrations of DBP appear to be able to rapidly absorb this chemical from the water, where it may interfere with normal biological functions.

The results of this work can also be applied retrospectively to the results of Chapter 2. Considering SPE was unable to recover an acceptable amount of DBP from the water samples in these experiments, that DMF appeared to be able to dissolve DBP as efficiently as methanol could, and that the time-lag between collection, extraction, and elution of these samples may have allowed time for the phthalates to be degraded suggest that poor analytical techniques obfuscated the results. It is, therefore, quite possible that the fathead minnows in the F_0 and F_1 generations of the pilot study were exposed to DBP concentrations closer to their nominal concentrations than originally concluded.

In terms of the current work, while no statistical analyses were conducted on the concentrations of DBP before and after the fish additions, our ability to duplicate these findings provides strong support for our hypothesis that DBP uptake in fish can be confirmed indirectly via the water. In both of the 100 μg DBP/L tanks used in the DBP-uptake experiments, the concentration of DBP declined markedly following the addition of fish. Further, in both experiments, the lowest measured concentration during the experiment was collected just one hour after the introduction of fish to the tank. Thus, it appears that the fish were capable of rapidly absorbing aqueous DBP.

The uptake of chemicals is widely documented in fish either via diet or the gills. Phthalates generally do not biomagnify via the food chain, probably as a result of their rapid metabolism in the gut (Mackintosh *et al.* 2004). DBP and its metabolites have been observed, however, to bioconcentrate in fathead minnow tissues at up to 2000 times their concentration in water (Call *et al.* 1983; Staples *et al.* 1997).

The most likely route for the uptake of these chemicals is via the gills, as it is for many chemicals with a log K_{ow} between 3 and 6 (McKim *et al.* 1985). Uptake at the gills is dependent, however, not only on the partition coefficient of a chemical, but on several additional factors. In simple terms, if a chemical can be dissolved aqueously, is smaller than 600 atomic mass units (amu), and can dissolve into the blood, then it is possible for a fish to absorb it via the gills (Randall *et al.* 1996). We can easily confirm that DBP is able to dissolve in water, and is likely to be able to pass through the gill epithelium as its molecular mass is only 278.35 amu. It is also likely DBP can dissolve into blood as the higher molecular weight phthalate, DEHP, has been frequently measured in whole (human) blood stored in blood bags, and DBP monoesters have been measured in human blood serum (Silva *et al.* 2007; Tickner *et al.* 2001). Other factors that affect the level of bioconcentration of any chemical include fish adipose tissue content, rate of oxygen uptake, and toxicant concentration in the water. Further, fish smaller than 4 g have markedly higher surface area to volume ratios so that cutaneous uptake should be considered in addition to uptake at the gills (Lien *et al.* 1994).

It would have been ideal to measure the DBP in fish tissues directly. However, we feel that the evidence presented here provides strong support for the ability of fish to rapidly take up DBP from water. There is a large body of evidence that suggests that fish can easily bioconcentrate phthalates including DBP, and also that decreasing concentrations of phthalates in water can be measured in parallel to their increasing concentrations in fish tissues. For example, a study conducted with radiolabelled DEHP (20 μg/L) in a static tank showed that juvenile rainbow trout (*Salmo giardneri*) in 3 L tanks rapidly absorbed the DEHP into their tissues, where concentrations reached over 10 μg/g (10 mg/kg) by 4 hours. These data were coupled with simultaneous water chemistry measurements of DEHP showing that as the concentration in the fish tissues increased, the aqueous concentration decreased. The DEHP concentration in the water fell to half of its original concentration after approximately 8 hours (n=4) (Tarr *et al.* 1990). The proportionally larger decrease in the concentration of DEHP in the water in Tarr *et al.* (1990) compared to my results, is likely due to the fact that this experiment was conducted in a static system. The use of a flow-through system, as used in the current work, involves the continual input of DBP into the system, and thus any decreases in aqueous concentration of DBP were mitigated by the inflow of more DBP into the tank. Considering this, however, the fact that uptake documented by Tarr *et al.* (1990) was much slower than it was in our experiment is surprising. We would expect that, in a static system, the decline in the aqueous concentration of DEHP would be even more rapid than in the current study. However, this disparity may be due to the nature of DEHP, which is a much larger molecule and is more lipophilic than DBP. This would likely slow the uptake of DEHP via the gills, based on its size, but on the other hand, it would be expected to have a higher affinity for lipid-rich blood based on its lipophilicity.

The main conclusions remain that DBP can be dissolved into water in a flow-through system consistently, at concentrations reasonably similar to nominal concentrations. Additionally, it is highly likely that fish are able to rapidly absorb DBP into their tissues via the gills and possibly also through the skin, since DBP concentrations in the tank water were repeatedly observed to decline rapidly following fish addition. Thus, we decided to move onto the next stage of this study: attempting to answer the original hypothesis that DBP causes anti-androgenic effects in fish at environmentally-relevant concentrations.

Chapter 4. Early life-stage exposure of the three-spined stickleback to di-*n*-butyl phthalate

4.1 Introduction

In the past few years the three-spined stickleback (*Gasterosteus aculeatus*) has emerged as a sensitive model for the assessment of endocrine disruption in fish. These fish are particularly useful for several reasons. They are small in size, have a short life cycle of 1-3 years, and are able to withstand variable conditions of salinity, temperature, pH, and oxygen concentration (Katsiadaki *et al.* 2007; Wootton 1984). Males and females can be differentiated from each other by molecular biology via a genetic sex marker gene (Peichel *et al.* 2004). The males also have androgen-dependent secondary sexual characteristics and behaviours, which are sensitive to endocrine disruption. Most importantly, three-spined sticklebacks are particularly useful in the study of endocrine disruption because they possess sensitive biomarkers for both oestrogenic and androgenic endocrine disruption; they have the capacity to produce vitellogenin, a yolk protein, in the liver when induced by oestrogens, and spiggin, a glue-like protein, in the kidney in response to androgens (Katsiadaki *et al.* 2007; Wootton 1984).

4.1.1 General biology of the three-spined stickleback

In the wild, three-spined sticklebacks have a widespread distribution throughout the coastal regions of North America, Asia, and Europe. They can be anadromous or purely freshwater, living in ponds, lakes, slow-flowing streams, and sheltered coastal bays. They are visual predators, feeding mainly on small aquatic invertebrates (Bell and Foster 1994; Ostlund-Nilsson 2007; Wootton 1984).

Three-spined sticklebacks are sexually dimorphic and gonochoristic; the sexes are genotypically and phenotypically separate throughout their lifespan (Griffiths *et al*. 2000; Lewis *et al*. 2008; Peichel *et al*. 2004; Wootton 1984). Adults are typically fairly small at maturity, reaching between 30 and 80 mm in length with three distinct dorsal spines (Wootton 1984) (Figure 4.1).

Figure 4.1. Drawings of three-spined sticklebacks. (a) An adult gravid female, and (b) a male in the breeding condition (sexual phase), displaying the bright red throat characteristic of the nuptial colouration. (Adapted from http: //pond .dnr .cornell .edu / nyfish/ Gasterosteidae /threespine _ stickleback _ pic .html).

4.1.1.1 Reproductive development

Three-spined sticklebacks are generally thought to take as little as 6-8 months to reach sexual maturity in the laboratory (Dr. I. Katsiadaki, personal communication). However, the cellular differentiation of the gonads begins much earlier. In fact, it is thought that by approximately 7 days post-hatch the gonads are identifiable as either ovaries or testes based on the degree of apoptosis and the number of primordial germ cells observed via histology. By 25-26 days after fertilization (17-18 dph) the sexes are even more distinct: females have developed primordial follicles in their ovaries, and males have cysts surrounded by somatic cells in their testes (Lewis *et al*. 2008).

In attempt to determine the period of reproductive development most sensitive to external disturbance in the three-spined stickleback, one study investigated the histological effects of either oestrogens or androgens on the gonads for varying periods of time after hatch. Some fish were exposed from hatching to 58 dph, some 0-14 dph, and others 14-58 dph. The researchers concluded that the first two weeks after hatch are considered to be a critical window for the feminization of the three-spined stickleback gonad, since 100% of the males had intersex gonads following exposure to oestrogen during this period. The masculinisation of the gonads is less well-understood. The authors concluded that, in males, the first 14 days post-hatch are critical, but this period of sensitivity may be longer (Hahlbeck *et al.* 2004). This corresponds well to studies of early life-stage endocrine disruption in the fathead minnow, in which the disruption of testicular development by 17α-ethinylestradiol was induced at any time between hatch and 20 dph (Van Aerle *et al.* 2002). Regardless, the critical window for the development of testicular tissue appears to be sensitive to disruption soon after hatch.

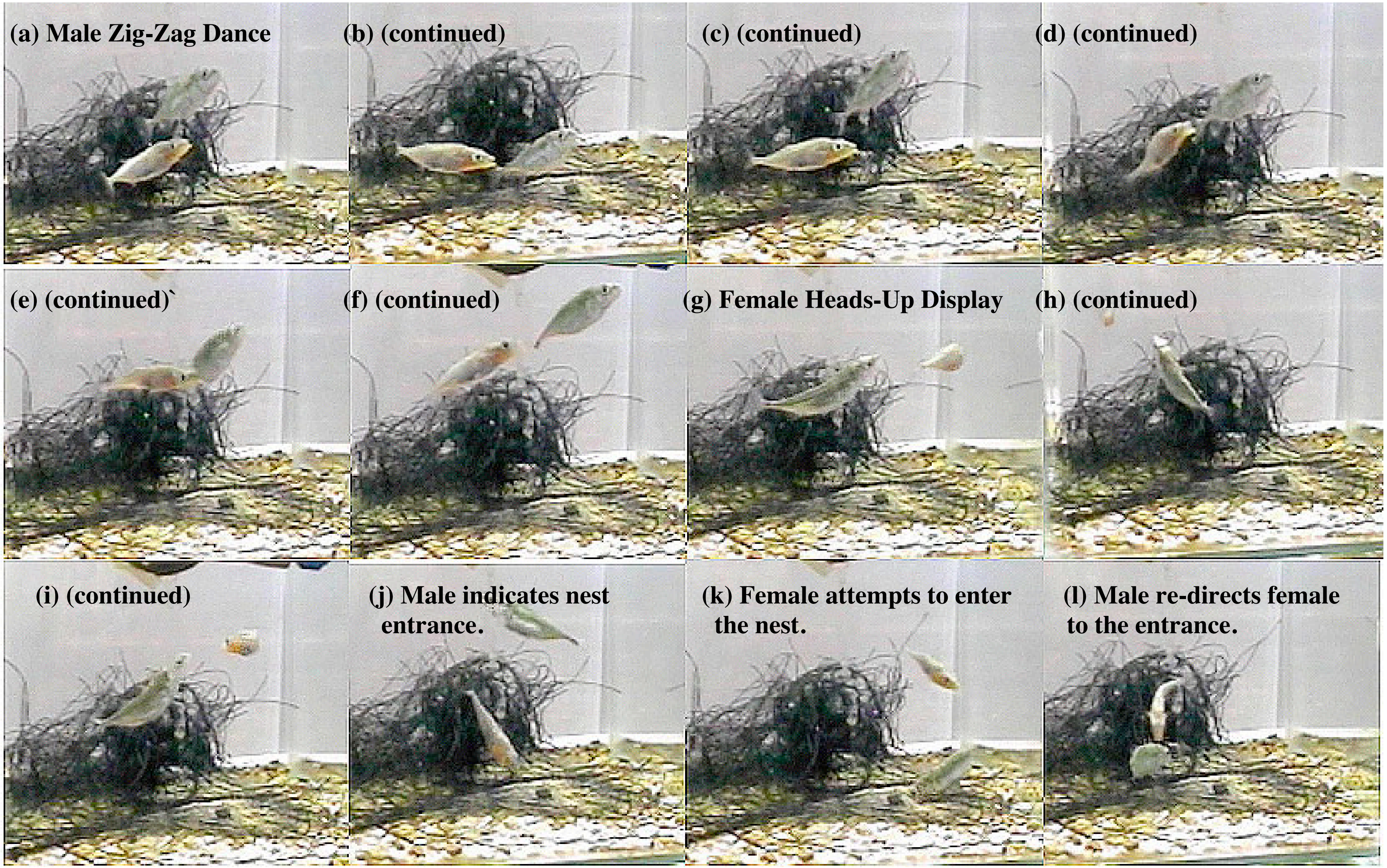
4.1.1.2 Spawning

Spawning in the three-spined stickleback in both the wild and in the laboratory occurs during a discrete spawning period and requires the development into a breeding condition. The timing of the entry into this sexual phase is highly dependent on increases in temperature, photoperiod, and light intensity, but is also likely influenced by innate cycles (Katsiadaki *et al.* 2007). Once induced by increasing temperature and longer day length, the female begins to produce vitellogenin and her eggs begin to mature in preparation for spawning, while the male produces higher concentrations of androgens, which stimulate behavioural, morphological, and physiological changes (Borg and Mayer 1995).

11-Ketotestosterone is thought to be the most important androgen controlling male secondary sexual characteristics and behaviours (Borg and Mayer 1995), but this point is currently under dispute. Testosterone, or even dihydrotestosterone, may be more important (Katsiadaki *et al.* 2007). Regardless, in preparation for spawning, the male undergoes several changes. He develops elaborate nuptial colouration to attract females, first, with the appearance of blueish eyes, followed by a red throat, and an iridescent green dorso-lateral area (Bakker 1994). Androgens also induce the production of spiggin in the kidney (Borg and Mayer 1995). Spiggin is a glue-like protein, excreted via the urogenital duct, that is used in nest construction. Finally, androgens also mediate the complex behaviours of nest-building, territoriality, and mating dances in males, necessary to attract female mates (Pall *et al.* 2002). These behaviours begin with the establishment of a territory. Nest-building ensues with males digging a small pit in the substrate and glueing bits of vegetation, sand and rocks to this pit with threads of spiggin, which is contained in a urinary bladder prior to its excretion. Eventually the male forms a small tunnel in the nest through which the female will pass to release her eggs. Once built, the male defends the nest from other male competitors.

The process of spawning involves several steps before a female will deposit her eggs. An ovulating female must first be coaxed towards the nest by a series of courtship (zig-zag) dances and displays of the nest by the male. If the female is willing, she will assume a “heads up” position by tilting her body at a 45° angle and showing the male her gravid belly. They then swim around one another, while he pricks at her belly with his spines. The male then shows her the nest, often "creeping" through it, depositing spiggin, and fanning it. When the female enters the nest, the male vigorously pokes at her sides in a quivering motion to encourage the release of the eggs. Once the eggs are released, she will exit the nest and the male quickly enters and fertilizes the eggs with his sperm. He then resumes territorial behaviour toward the female and chases her away (Figure 4.2). Over the next 5-12 days, the male fans the nest by the rapid movement of his pectoral fins, a behaviour which is not androgen-mediated (Ostlund-Nilsson 2007; Pall *et al.* 2002; Wootton 1984). This aerates the eggs with oxygen as they develop.

In the wild, breeding typically takes place between March and early August, but timing can vary with latitude (Wootton 1984). In the laboratory, spawning can be induced earlier in the year using changes in photoperiod and light intensity, but the mature sticklebacks will inevitably come into breeding condition by spring, regardless of the laboratory conditions. Generally, with the onset of increasing temperatures and photoperiod, female sticklebacks require two to three months to become fully sexually mature and able to ovulate. The males, on the other hand require 3 to 6 weeks to become ready to spawn once induced (Katsiadaki *et al.* 2007).



(a) Male Zig-Zag Dance

(b) (continued)

(c) (continued)

(d) (continued)

(e) (continued)`

(f) (continued)

(g) Female Heads-Up Display

(h) (continued)

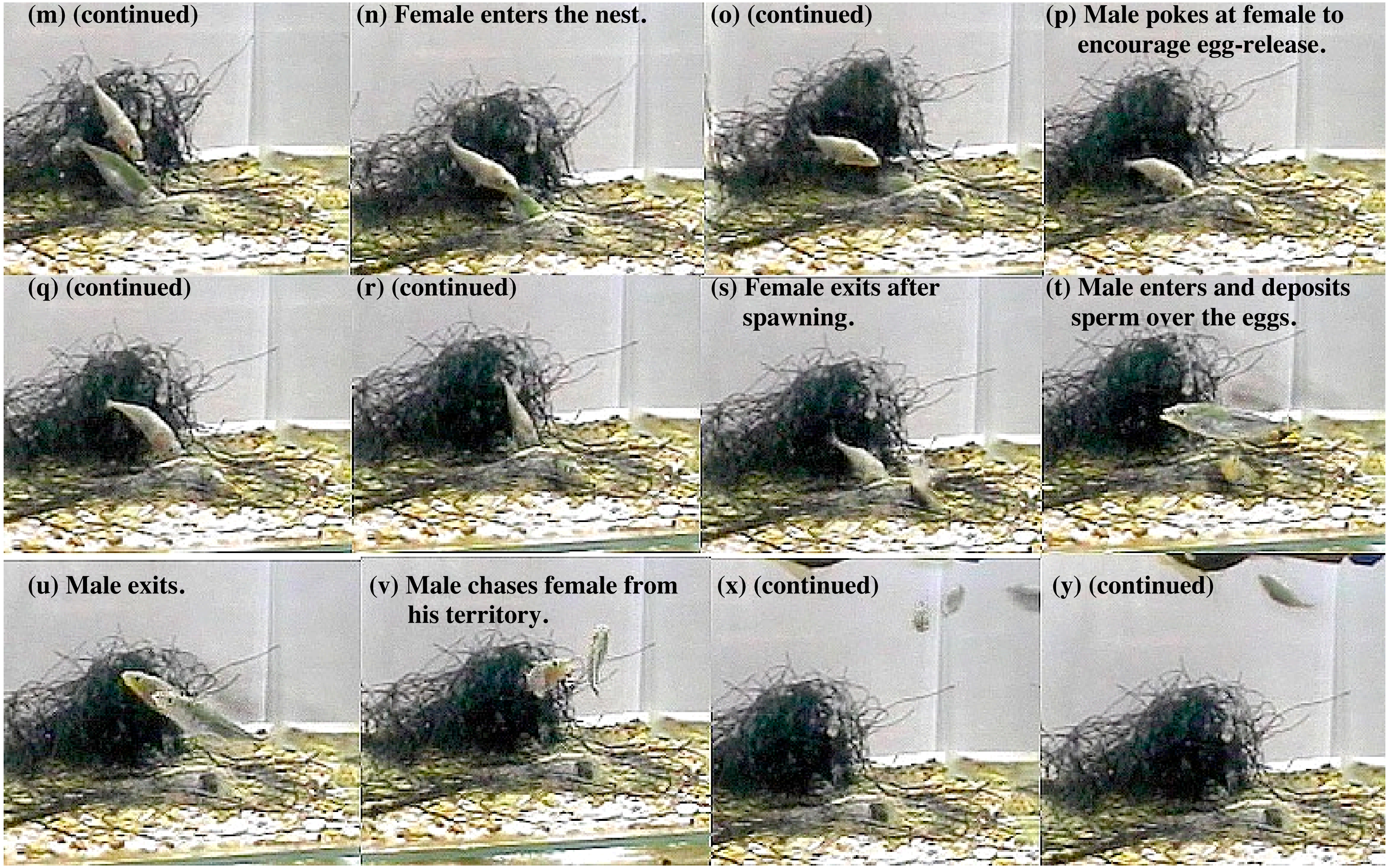
(i) (continued)

(j) Male indicates nest entrance.

(k) Female attempts to enter the nest.

(l) Male re-directs female to the entrance.

Figure 4.2. Photographs of a pair of three-spined sticklebacks during courtship and spawning. (See captions for a description of each photograph). Photographs by K. Aoki, Institute for the Environment.



(m) (continued)

(n) Female enters the nest.

(o) (continued)

(p) Male pokes at female to encourage egg-release.

(q) (continued)

(r) (continued)

(s) Female exits after spawning.

(t) Male enters and deposits sperm over the eggs.

(u) Male exits.

(v) Male chases female from his territory.

(x) (continued)

(y) (continued)

Figure 4.2. (continued).

4.1.2 Experimental aim and design

The experimental aim of this study was to investigate whether or not exposure to DBP during early development, the first 24 days post-hatch, would significantly affect the gonadal histology, plasma androgen concentrations, nuptial colouration, spiggin concentrations, and reproductive behaviour and success of three-spined sticklebacks at maturity.

This exposure period was chosen due to the higher sensitivity of young versus adult mammals to the effects of phthalates (Wine *et al.* 1997). Thus, it was considered important to investigate the effects of DBP on fish during a similar point in their life cycle. However, due to the lack of a discrete period of sensitivity for the masculinization of the gonads reported by Hahlbeck *et al.* (2004), the exposure period was extended from 14 days post-hatch to 24 dph.

In order to test the effects of DBP on the secondary sexual characteristics and courtship behaviours of male sticklebacks, it was also necessary to consider the confounding effects that dominance hierarchies and competition might have on the expression of such traits (Candolin 2000). To control these factors, a nesting trial was designed to enable the anti-androgenic effects of DBP on males induced into breeding condition to be measured, while free from competition. This design was based on the work of Dr. Ioanna Katsiadaki at CEFAS, Weymouth (Katsiadaki *et al.* 2007).

4.2 Methods

This study was conducted from August 2008 to June 2009 and consisted of three experiments, each based on separate batches of eggs. The methods varied slightly between each experiment. In general, the eggs for each experiment were divided evenly between three treatment groups around the time of hatch. They were then exposed to different treatments until 24 dph, including a Solvent Control and various concentrations of DBP. After the 24 days, chemical exposure was terminated, and the fish were left to grow for approximately 5-8 months in clean water. Fish were sampled on three different occasions from each experiment, at 130 DHP, 180 dph, and following a nesting experiment (Figure 4.3). The differences are also outlined in detail below (Table 4.1).

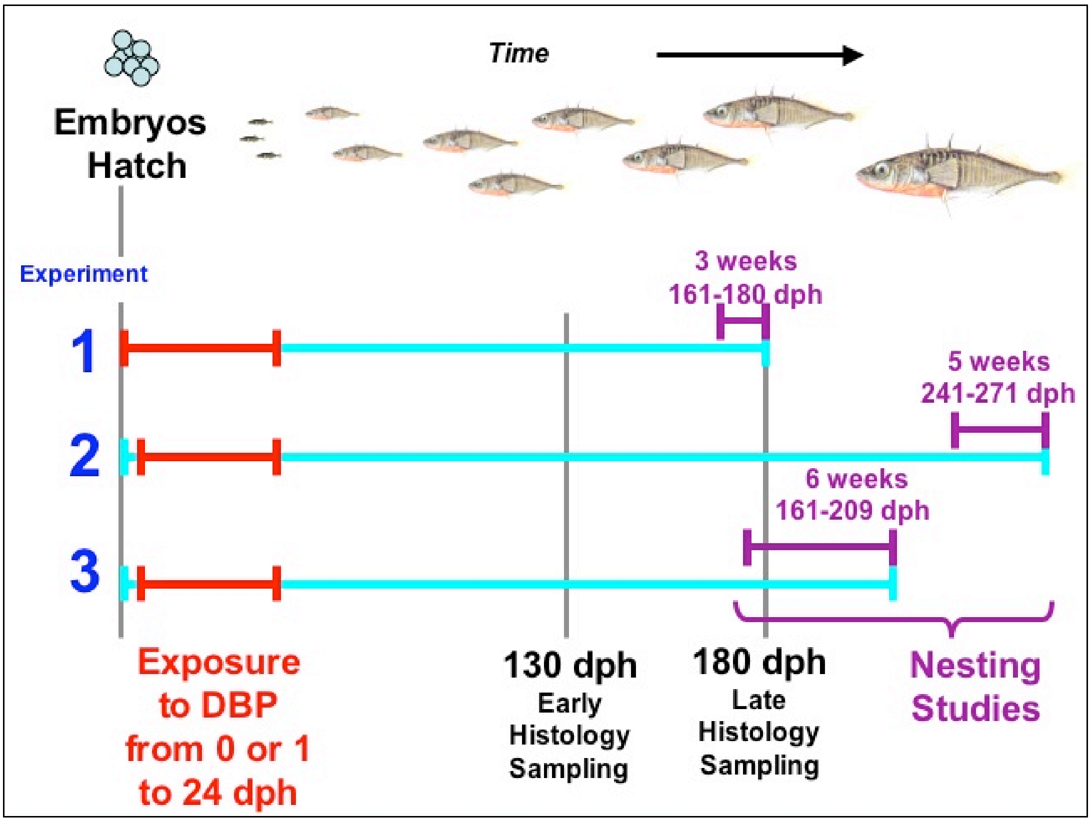


Figure 4.3. Depiction of the differing timelines for Experiments 1 to 3, exposing three-spined sticklebacks to various concentration of DBP 0 or 1-24 dph and allowing them to grow into maturity.

Table 4.1. The differences in the methods of fertilization, the parameters sampled at different time points, the age of the fish in the nesting studies, and whether or not spawning success was measured in Experiments 1 through 3, which were exposed to DBP during early life.

Experiment	Treatment (Nominal Concentration)	Method of Egg Fertilization	Exposure Period	Parameters Measured in Fish Sampled at 130 dph	Parameters Measured in Fish Sampled at 180 dph	Age During Nesting Study	Length of Nesting Study	Allowed to Spawn	Parameters Analysed in Nesting Fish
1	Solvent Control	Natural fertilization in nest	0-24 dph	- Histology - 11-KT Concentration[†]	- Histology - 11-KT Concentration[†]	161-180 dph	3 wks	No	- Concentrations of T, 11-KT, and E2[†] - Nuptial colouration - Nest building - Spiggin concentration
	50 μg DBP/L			Not sampled					
	100 μg DBP/L—1			- Histology - 11-KT Concentration[†]					
	100 μg DBP/L—2		1-24 dph	Not sampled					
2	Solvent Control	*In vitro* fertilized	1-24 dph	- Histology - 11-KT Concentration[†]	- Histology - 11-KT Concentration[†]	241-271 dph	5 wks	Yes	- Histology - Concentrations of T, 11-KT, and E2[†] - Nuptial colouration - Nest building - Kidney Cell Height - Spawning success
	50 μg DBP/L								
	100 μg DBP/L								
3	Solvent Control	*In vitro* fertilized	1-24 dph	Not sampled	- Histology - 11-KT Concentration[†]	161-209 dph	6 wks	No	- Concentrations of T, 11-KT, and E2[†] - Nuptial colouration - Nest building - Spiggin concentration
	100 μg DBP/L								
	200 μg DBP/L								

[†] Refers to plasma concentrations of testosterone (T), 11-ketotestosterone (11-KT), and/or oestradiol (E2) by radioimmunoassay.

4.2.1 Exposure system

The exposure system for all three experiments consisted of ten 8 L tanks randomly assigned to a treatment. The treatments included Solvent Controls (methanol), Low Dose (50 μg DBP/L), High Dose (100 μg DBP/L), and Extra High Dose (200 μg DBP/L) tanks. The water was cooled using a titanium immersed-coil chilling unit to 18-20°C. All tanks were part of a flow-through system similar to the one described in Chapter 2, Section 2.2.2. (Figure 4.4).

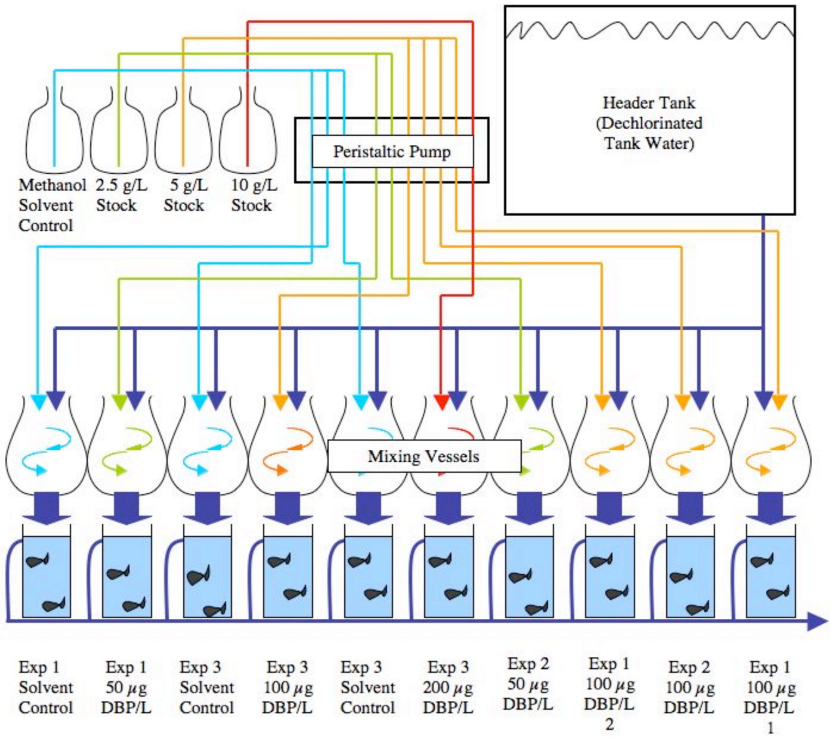


Figure 4.4. Diagram of the flow-through system for the exposure of three-spined stickleback fry from Experiments 1, 2, and 3 to various concentrations of DBP from 0-24 dph.

The flow rates of both the incoming water and the DBP-stock solutions were checked every 2-3 days. The water was collected from the outflow of each tank with a large graduated cylinder in order to measure the water volume after 1 minute. The flow rate was set at 500 ml/min. The stock solution flows were measured over 10 minutes, for an expected rate of 0.01 ml stock/minute.

The photoperiod mimicked the natural summer conditions for three-spined sticklebacks, with 16h light: 8h dark. Light intensities were a minimum of 500 lux, but usually between 600 and 1800 lux. All tanks contained 0.5 cm gravel on the floor of the tank and one inverted piece of a semi-circle of ABS piping to provide some shelter for the growing fish.

All fish were exposed to DBP from either 0 or 1 dph to 24 dph: Experiment 1 from August 6th to 29th, 2008; Experiment 2 from August 29th to September 22nd, 2008; Experiment 3 from September 2nd to 26th, 2008.

4.2.1.1 Water chemistry

All tanks were sampled approximately every 4-7 days for the duration of the exposure periods. Each tank was sampled in duplicate. Using clean, rinsed volumetric flasks, 500 ml were collected from each Solvent Control and 50 μg DBP/L tanks, and 250 ml were collected from the 100 and 200 μg DBP/L tanks. All water samples were kept on ice and extracted within 3 hours of collection. Each water sample was decanted into a Separation Funnel and liquid-liquid extracted three times with dichloromethane (DCM) by shaking it vigorously for 1-3 minutes according to the protocol outlined in Table 4.2.

Table 4.2. Volumes of dichloromethane and extraction times used in the liquid-liquid extraction protocol for water samples collected from the fish tanks in the experiments exposing three-spined sticklebacks from 0-24 dph to various concentrations of DBP.

Nominal Concentration in Tank	Volume of Water Sample	Extraction 1		Extraction 2		Extraction 3		Total Volume of DCM Extract (ml)
		Volume of DCM (ml)	Time (min)	Volume of DCM (ml)	Time (min)	Volume of DCM (ml)	Time (min)	
Solvent Control	500x2	20	3	20	3	10	1	50
50 μg DBP/L	500x2	20	3	20	3	10	1	50
100 μg DBP/L	250x2	10	3	10	3	5	1	25
200 μg DBP/L	250x2	10	3	10	3	5	1	25

The extracts were dried under a stream of nitrogen, reconstituted in 1ml hexane, stored at 4°C, and run on the Clarus 200 GCMS (Perkin Elmer Ltd.) along with a set of phthalate standards according to the parameters described in Chapter 2, Section 2.2.6.2.

4.2.2 Source of stickleback embryos for Experiments 1 through 3

4.2.2.1 Experiment 1

Experiment 1 eggs were produced by 7 pairs of three-spined sticklebacks in the laboratory of Dr. Ioanna Katsiadaki at the CEFAS in Weymouth, UK by natural spawning. The males built nests from filamentous detritus, such as algae and small sticks, and females spawned with males in these nests on July 28th, 2008. The egg-bearing nests were collected five days later on Friday August 1st, 2008 and transported to Brunel University. During transport, the nests were submerged in dechlorinated water with air circulation provided by a battery-powered air pump. The water was treated with a nominal concentration of 1 μg/L methylene blue (Sigma-Aldrich Ltd.) to prevent fungal growth in the nests. The container was surrounded by ice, to cool the water (10-20°C). Transport took approximately 3 hours.

Upon arrival, we trickled laboratory tank water into the container holding the nests to acclimatize them to the water temperature in the laboratory (18°C). Once acclimatized, the nests were gently transferred with gloved hands to the flow-through treatment tanks: Solvent Control, 50 μg DBP/L, and 100 μg DBP/L. Two nests were added to each of the Solvent Control and 50 μg DBP/L tanks, and three nests were placed in the 100 μg DBP/L tank (Figure 4.5a). Again, the three exposure tanks had been dosed with the appropriate concentration of DBP for 3 days prior to the a of the egg-bearing nests.

A fourth treatment tank was assembled after several fry were discovered to have hatched in the container used to transport the nests from CEFAS to Brunel University. This tank was dosed at 100 μg DBP/L, and named "100 μg DBP/L—2". These fish were exposed to DBP for 1 day less than the other fry in Experiment 1. Miscommunication resulted in two more unexposed fry from this transport container being added to the 100 μg DBP/L—2 tank four days later, resulting in two unidentifiable fish (amongst the others) being exposed starting only 4 dph.

4.2.2.2 Experiments 2 and 3

Eggs for Experiments 2 and 3 were produced by *in vitro* fertilization (IVF), on August 20th and 25th, 2008, respectively, at CEFAS Laboratories in Weymouth. The use of IVF is beneficial as it produces fry that are less variable because maternal effects (eg. egg size) and parental care (eg. fanning of the nest) are no longer confounding factors (Barber and Arnott 2000).

This involved the termination of a mature male. The testes were then excised, homogenized, and suspended in 900 μl Hank's solution, which was prepared as follows:

- 1.0 ml stock 1 (8 g NaCl, 0.4 g KCl, 100 ml dH_20)
- 0.1 ml stock 2 (0.358 g Na_2HPO_4 (anhydrous), 0.6 g $KHPO_4$, 100 ml dH_20)
- 0.1 ml stock 3 (0.72 g $CaCl_2$, 50 ml dH_20)
- 8.6 ml dH_20
- 0.1 ml stock 4 (1.23 $MgSO_4X7H_2O$, 50 ml dH_20)
- 0.1 ml of freshly prepared stock 5 (0.35 g $NaHCO_3$, 10 ml dH_20)

An ovulating female was stripped of her eggs by gently squeezing her lateral sides towards her urogenital opening. Approximately 25 μl of testes homogenate was pipetted over roughly 150 eggs in a sterile Petri dish. Within 15 minutes, the fertilized eggs could be identified under a dissecting microscope by a thickened membrane. These eggs were placed in an Erlenmeyer flask containing tank water and dilute methylene blue (1 μg/L).

Transportation of the eggs occurred on August 26th, 2008, over 3 hours. The flasks were aerated with the battery-powered air pump and surrounded with ice packs in a Styrofoam container. Upon arrival, the flasks were removed from the container, placed under bright lights (>500 lux), and aerated until hatch. Fresh tank water flowed into the flasks approximately one day before hatch to dilute out the methylene blue (Figure 4.5b).

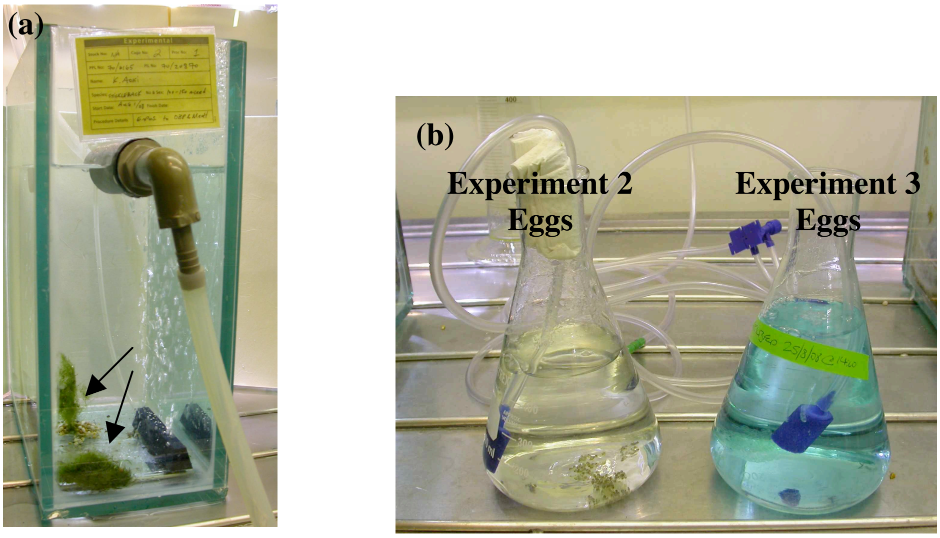


Figure 4.5. Photographs of the (a) 50 μg DBP/L tank from Experiment 1 containing two nests (arrows) , and (b) Experiment 2 and 3 eggs (left and right, respectively) before hatch. Experiment 2 eggs are in fresh water and Experiment 3 eggs are in fresh water with dilute methylene blue to prevent fungal growth.

4.2.3 Animal husbandry

4.2.3.1 Experiment 1

Experiment 1 eggs were allowed to develop without interference, but the tanks were observed daily for signs of development, fungal infection, and hatching. On August 5th 2008, at least one hatched fry in each tank was observed. All fish had appeared to have hatched by 10:00 A.M. on August 6th, 2008, and this was considered to be 0 dph for Experiment 1 fish. Again, the treatment tanks in this experiment were the Solvent Control, 50 μg DBP/L, and 100 μg DBP/L. The number of fry in each treatment tank could not be assessed since they could not easily be counted at such an early stage. However, on October 27th, 2008 there were 48, 4, 48 and 20 fish in each of the Solvent Control, Low, High—1 and 2 tanks, respectively

(Table 4.3 in Section 4.3.2.2). The filamentous nesting material was removed at 5 dph, since it contained some fungal growth, posing a threat to fry health.

4.2.3.2 Experiments 2 and 3

Experiment 2 and 3 eggs were left in the Erlenmeyer flasks with clean tank water until the majority of Experiment 2 fry had hatched by Friday, August 29th, 2008, and all of the Experiment 3 fish had hatched by September 2nd, 2008.

In both experiments, the flask was suspended in a container with flow-though tank water and allowed to acclimatize to the tank water temperature for approximately 4 hours. Once acclimatized, the fish were gently sucked up by a plastic pipette and divided sequentially into their treatment tanks (eg. the first fish was placed in the Solvent Control tank, the second in the 50 μg DBP/L tank, and the third in the 100 μg DBP/L tank, and this was repeated until all fish had been placed in their treatment tanks).

In Experiment 2, approximately 185 hatched fry and some unhatched embryos were divided among the three treatment tanks: Solvent Control, 50 μg DBP/L, and 100 μg DBP/L, giving about 60 fry per treatment. In Experiment 3, approximately 45 fry were added to each of the three treatment tanks: Solvent Control, 100 μg DBP/L, and 200 μg DBP/L (Table 4.3 in Section 4.3.2.2). Exposure to DBP in both experiments was considered to have started at 1 dph.

4.2.3.3 Feeding

From 3 dph, fry were fed with hatched *Artemia* nauplii (brine shrimp) three times daily. Chopped up blood worms (*Chironimid* larvae) were incorporated into the feeding regimen at approximately 50 dph. When the fish were large enough to properly digest full-sized blood worms, feeding switched completely to blood worms, three times daily. At approximately 100 dph, fish from all three experiments were fed four times daily: twice with blood worms and twice with ZX 400 Fish Food (ZM Systems Ltd.).

4.2.4 Post-exposure conditions

Upon termination of the DBP-exposure period (at 24 dph), the fish were transferred to clean, aerated tanks flowing with water at a rate of 20 L/hour. In Experiment 1, to maintain similar densities and growth rates across all of the tanks, the treatment groups with fewer fry were placed in 8 L tanks while the treatment groups with more fish were transferred to 12 L tanks.

At the termination of the Experiment 2 exposure period, both the fish in Experiments 1 and 2 were transferred to 40 L tanks to encourage growth (except for the 50 μg DBP/L in Experiment 1 tank because it contained only four fish). At the end of the Experiment 3 exposure period, the fish from this experiment were also transferred to 40 L tanks (Figure 4.6).

Figure 4.6. Photograph of the laboratory containing the tanks of Experiments 1-3. Each 40 L tank contained three-spined sticklebacks in clean tank water, which had previously been exposed to various concentrations of DBP for 0-24 dph.

Due to uncontrollable events, the laboratory water supply was switched several times during the post-exposure period of the fish. The water supply was shut off nightly (17:00 to 10:00) from October 8th to 10th, and 13th to 15th, 2008, due to construction work. The water source was changed permanently on October 15th, 2008, from a reservoir to a direct connection with the main water supply. Action was taken to ensure the incoming water was properly de-chlorinated using carbon filters. Nitrate, nitrites, and ammonia were monitored daily during these periods, and all remained low.

The numbers of fish in each tank were counted when they were large enough size to be counted (October 27th, 2008). On December 10th, 2008 (at 95-122 dph), steps were taken minimise the differences in density between treatment groups. This was adjusted by changing the tank size so that the difference in water volume per fish was no larger than ±120 ml within each experiment. Overall, the fish density between all experiments ranged from 0.7 to 1.63 fish/L.

Over 3 days starting December 16th, 2008, the photoperiod was reduced to 8h light: 16h dark, to mimic winter conditions in all fish tanks. Light intensity was also reduced at this time to low levels, ranging from 58 to 350 lux. This was conducted to ensure that the male fish would not be encouraged to enter the breeding condition prior to conducting the nesting trials which aimed to test the timing of the entry into this breeding state induced by light.

4.2.5 Early sampling of fish at 130 days post-hatch

In Experiments 1 and 2, some fish were sampled at approximately 130 dph, (December 15th, 2008 and January 6th, 2009, respectively). In Experiment 1, only fish from the Solvent Control (n=15) and the 100 μg DBP/L—1 (n=16) groups were sampled; the 50 and 100 μg DBP/L—2 tanks were not sampled because they contained smaller numbers of fish (Table 4.1; Table 4.3 in Section 4.3.2.2). The Experiment 2 fish were sampled from all treatment groups (n=18). Fish from Experiment 3 were not sampled because of the small number of fish in the Solvent Control (Table 4.1; Table 4.3 in Section 4.3.2.2).

The fish were then sampled, according to the methods described in Chapter 2 Section 2.2.7. In brief, they were anaesthetized and pithed. Blood was sampled by removing the caudal fin and collecting the blood in a heparinized capillary tube, which was decanted into a clean eppendorf tube, and stored on ice. Fork length and weight were measured. The caudal fin was weighed, placed in a sterile tube, and snap frozen in liquid nitrogen. The body cavity was opened with sterile, RNAse-free utensils to identify sex, and the whole fish was either snap frozen in liquid nitrogen or placed in Bouin's solution for 24 hours. The fin and whole-body samples in liquid nitrogen were transferred to a -80°C freezer for storage. The histology samples were switched from Bouin's solution to 70% IMS after 24 hours, and again after 48 hours.

The plasma samples were processed as in Chapter 2, Section 2.2.7. In brief, they were centrifuged at 12,500 g for 5 minutes within 3 hours of collection. Aliquots of 2, 4, 6, 8 μl or more were drawn off into clean tubes. Ethyl acetate was then added to each tube containing plasma. This time the plasma to ethyl acetate ratio used was 1:220. Once added the samples were vortexed and stored at -20°C.

4.2.6 Late sampling: Non-nesting fish at 180 days post-hatch

The fish that were not used in the nesting trials for Experiments 1 through 3 were all terminated at 180 dph (February 2nd, 25th and March 3rd 2009, respectively) (Table 4.1). These three-spined sticklebacks were sampled for blood, length and weight, genetic sex, and histology, as described in Section 4.2.5.

4.2.7 Nesting and spawning experiments

Three nesting trials were conducted between January 13th and June 9th, 2009, with the fish from Experiments 1-3. Each nesting trial varied slightly (Figure 4.3; Table 4.1), and differences will be discussed separately, but in general it involved testing whether or not early life-stage exposure to DBP inhibited the onset of the development of breeding condition in males exposed to increased light intensity and photoperiod (Katsiadaki *et al.* 2007). These studies were conducted with fish placed in individual tanks, free from competition.

The nesting tanks were set up at least 24 hours prior to the addition of fish, in order to flush out the system. Once added, the fish that had been kept in "winter" condition (8h light:16h dark, with low light (<500 lux)), were induced into breeding condition by exposing them to “summer” conditions (16h light:8h dark and >500 lux of light intensity). They were allowed to experience these conditions for 10 days, after which nesting material was introduced into their tanks. The nesting material consisted of approximately 50 ml of clean gravel, 30 ml of silica sand, and a handful of black and green polyester thread (3-5 cm in length) along with one red thread (5 cm in length). Females are very attracted to the colour red during mating, and thus the males often use this colour thread to mark the entrance to the nest, which aided us in the identification of nests during the study. On Day 10, fish were also visually isolated by inserting opaque dividers between all tanks.

On Day 16, a large wild-caught female was put into visual contact with each fish to encourage nest-building and/or maintenance. This was conducted by placing females into 600 ml beakers with clean tank water. These beakers were randomly distributed between tanks, and left for approximately 5 minutes, to provide a visual stimulus for the nesting males (Figure 4.7). This was repeated occasionally throughout the remainder of the experiment. Nest-building activity was monitored by taking a photograph of each tank every second day, starting on Day 10.

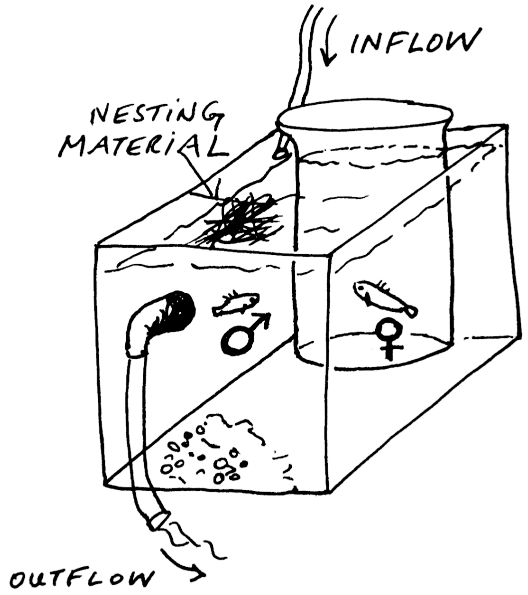


Figure 4.7. Depiction of the addition of a female three-spined stickleback to a nesting tank containing a male. The female was held in a glass beaker to separate her from the nesting male.

Sampling was conducted at the end of every nesting study following the methods outlined in Section 4.2.5, except that a photograph of the lateral and ventral parts of the head of each fish was taken prior to the collection of blood for the analysis of nuptial colouration.

The photographs of each male three-spined stickleback were taken using a 6.1 megapixel digital camera (D40, Nikon Ltd.) with a macroscopic lens. Following anaesthetization, each male was submerged in tank water in a black wax-bottomed dissecting tray. The camera was suspended above the tray with a tripod and two heat-free light sources were pointed approximately 45° from the fish to prevent shadows. A ruler was also in the frame to standardize any size differences (Figure 4.8). A photograph was taken of both the ventral and right sides of each fish (Figure 4.9).

Figure 4.8. The set-up for photographing male nuptial colouration in male sticklebacks following a nesting trial.

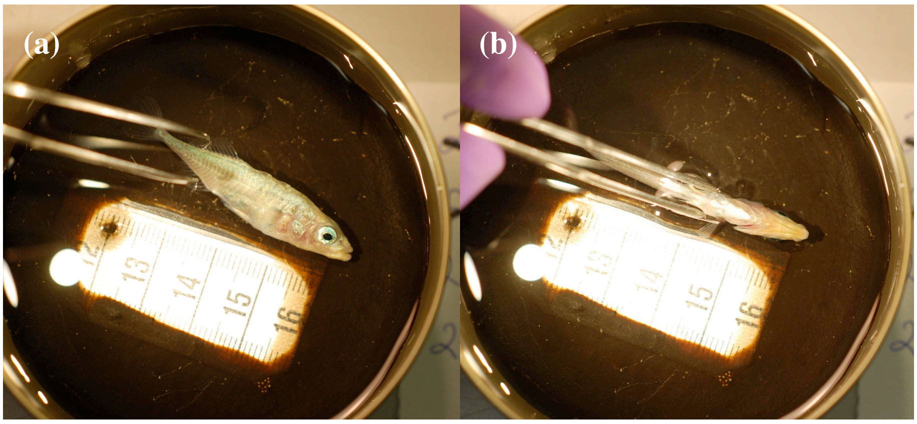


Figure 4.9. Photographs of (a) lateral and (b) ventral sides of a male three-spined stickleback used in the nesting trials.

In Experiments 1 and 3, after the photograph of each fish had been taken, the blood was sampled, the length and weight were measured, and the kidney and gonads were carefully excised, making sure not to burst the spiggin-filled urinary bladder (Figure 4.10). These organs were then weighed, and placed in sterile 1.5 ml tubes. The gonads were snap frozen in liquid nitrogen, then stored at -80°C for molecular analysis, and the kidneys were placed on ice and stored at -20°C for analsis of spiggin. In the Experiment 2 nesting study, the fish were left intact and placed in Bouin's for histological analysis, as described before (Section 4.2.5).

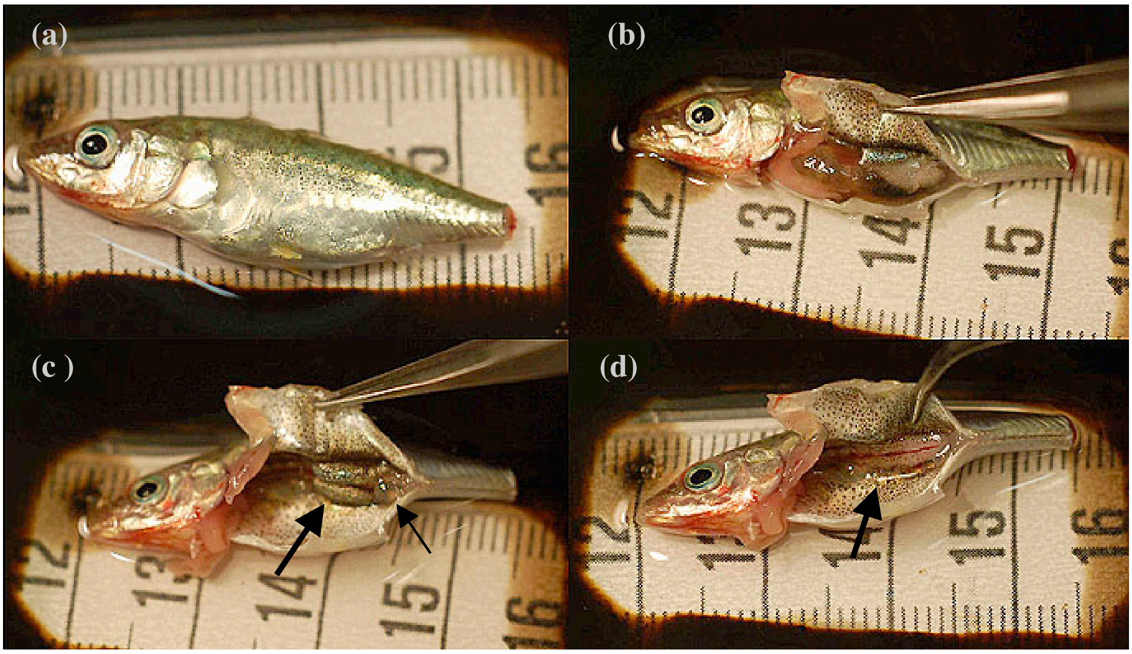


Figure 4.10. Photographs of the dissection of a male three-spined stickleback from Experiment 1. (a) The body is intact, but the tail has been removed, (b) the body cavity has been opened ventrally to show the viscera and air bladder, (c) the viscera have been removed leaving the testes (large arrow) and urinary bladder (small arrow), and (d) the gonads have been removed to show the kidney lying on the dorsal body wall (arrow).

4.2.7.1 Experiment 1 nesting protocol

The nesting experiment for Experiment 1 was conducted over 21 days from 160 – 181 dph (January 13th, 2009 to February 2nd, 2009). Thirty-six tanks were assembled in blocks of twelve, half of which were elevated in the back row of tanks (Figure 4.11). Since sex was unknown, twelve fish from each treatment group were randomly selected and each placed into one of the twelve nesting tanks for that group.

Figure 4.11. Photograph of the experimental system for the nesting trial. Each tank contained one randomly-selected Expriment 1 three-spined stickleback from the Solvent Control, 100 μg DBP/L—1 or 2 treatment groups arranged in blocks of twelve tanks.

On Day 10, the nesting material was added, the fish were monitored, and photographs of each tank were taken every second day to monitor nest-building progress. On Day 16, the fish were shown a female for 5 minutes each as described in Section 4.2.7. On Day 21, all fish were sampled.

4.2.7.2 Experiment 3 nesting protocol

Based on the experiences gained from the first nesting trial with the Experiment 1 fish, some adjustments in the protocol were made. Firstly, the nesting trial using fish from Experiment 3 was extended to 6 weeks (February 16th to March 30th, 2009; 161 – 209 dph). Secondly, all tanks were placed at the same height, so as to minimize any potential differences in light intensity between tanks. Again, twelve fish were randomly selected from each treatment group (Solvent Control, 100 μg DBP/L, and 200 μg DBP/L) and added to individual tanks within each block of twelve tanks. On Day 10, the nesting material was added, as described in Section 4.2.7.1, and the dividers were moved to separate the fish from one another.

It should be noted that on Day 10, the fish in tank 29 (a female from the 200 μg DBP/L treatment group) was noted to have bubbles located subdermally near the caudal fin. This was diagnosed visually as Bubble Disease, due to supersaturated oxygen in the warmed water (Figure 4.12). Air stones were placed in the header tanks to encourage the precipitation of oxygen from the water. Over the next two days the bubble on the fish shrunk and the fish appeared fine thereafter, as did the oxygen concentration.

On Day 17, six wild-caught gravid females were shown to the nesting fish for 5 minutes each (Figure 4.7). This was repeated on Days 19 and 34 and the fish were sampled on Day 43, as described previously.

Figure 4.12. The fish from Experiment 3 with Bubble Disease on its tail (arrow), photographed on February 25th, 2009.

4.2.7.3 Experiment 2 spawning protocol

By March of 2009, despite maintenance of the three-spined sticklebacks in "winter" conditions, the Experiment 2 males in all of the treatment tanks appeared to be reaching the breeding state. This was characterized by the appearance of blue eyes, and red colouration on the throats of the males. Since it was now possible to distinguish the males from the females, only the fish displaying this colouration were chosen for this nesting trial. This nesting experiment also differed from the previous nesting experiments that, in addition to monitoring nesting behaviour, I tested whether or not the three-spined sticklebacks exposed to DBP were able to spawn and produce viable offspring (Table 4.1). Thus, all of the fish from Experiment 2 also had to be put into "summer" conditions, so that the females would be induced to ovulate, and thus, spawn.

Eight males were randomly placed into eight individual tanks per treatment group. To select the males for the nesting trial, all of the males displaying some nuptial colouration from each treatment group were caught and placed in a large container. A table of random numbers was then used to allocate eight fish from each treatment group to one of the 24 tanks by the order in which they were caught. The treatment group to which each fish belonged was marked on each tank. This randomization was introduced to help minimize variation in lighting and/or disturbance (eg. proximity to the door), which was not conducted in the other nesting trials.

The females remained in the three larger group tanks for the duration of this study. Any males in these group tanks were removed. The spawning study was run for 5 weeks but the hatched fry were allowed another week to grow, thus the entire experiment ran from April 27th to June 9th, 2009 (fish were 241 – 271 dph). As before, the photoperiod was increased over the course of two days to summer conditions in both the nesting tanks and also in the tanks containing the females.

In the nesting tanks, visual contact was maintained between all nesting tanks until Day 10, when nesting material was added. On Day 17, the spawning attempts were begun and spanned 15 days. Each male was allowed to attempt to spawn until they did so, or until Day 31, whichever came first.

Each day spawning was attempted, males were given three opportunities to spawn with three different females. Spawning attempts involved the addition of a female from the same treatment group for a maximum of 20 minutes. If, after 1-2 minutes, it was clear the female was not interested in spawning she was removed, as the males were quite aggressive towards unreceptive females. If she displayed interest, she was generally left in the tank, as it could take up to 20 minutes for the pair to spawn. Female interest was evidenced by the head-up display. Following the various dances of the male and female, spawning was confirmed if:

- the female entered the nest at the appropriate end.
- the male conducted prickling, rapidly poking at the caudo-lateral area of the female.
- the female left the nest with a clearly flaccid belly.
- the male entered the nest afterward to deposit his sperm (Figure 4.2).

Once a pair had spawned, the female was removed and sampled immediately for blood, length, weight, and histology, according to the methods described previously in Sections 4.2.5 and 4.2.7. The male was allowed to tend to the eggs until they hatched. Upon hatch, the male was removed as soon as possible to prevent consumption of the fry. The newly hatched fry were left to grow for 5-8 days, to see if they were able to survive after the resorption of their yolk sac, which occurs ~3-4 dph. Adult males were sampled immediately following their removal for nuptial colouration, blood, length, weight, and histology. All males and females that had failed to spawn were sampled in the same manner on Day 31. The fry were left in the tanks and fed with Liquifry (Tetra Ltd.) for 5-8 days until they were terminated.

4.2.8 Sample processing methods

4.2.8.1 Histology

The methods used to process the histological samples are described in Chapter 2 (Section 2.2.9). Additionally, due to the hard plates on the lateral sides of the three-spined stickleback (Wootton 1984), a decalcification step was added. This step, using a strong acid and chelating agent, dissolves the plates so that they do not interfere with histological processing. This was conducted prior to tissue processing, between the first and second step of dehydration in 70% IMS.

Once prepared, the histology samples were placed in Decalcifier II (Surgipath Europe Inc.) at a 1:20 ratio (volume of tissue to decalcifying solution) for two hours. This solution was decanted, and the samples were placed in fresh 70% IMS to await processing.

The histological slides were analysed with a compound light microscope according to EPA (2009). While not identical, the fathead minnow gonads are histologically quite similar to those of the three-spined stickleback. The guidelines were very useful in the identification of abnormalities, and standardization of histological analysis. In general, histological analysis was conducted in females and males separately. In both cases, initial analysis was not conducted blindly. The samples analysed first were the Solvent Controls, followed by the samples from the fish exposed to the highest concentrations of DBP, and finally by the fish exposed to lower DBP concentrations. This order was used to familiarize the examiner with the "normal" histology of the stickleback in the Solvent Controls, and to better identify any anomalies by then analysing the fish exposed to the highest concentrations of DBP. To minimize bias, any abnormalities identified in this initial inspection were re-analyzed blindly in all of the samples.

4.2.8.2 Identification of genetic sex

Fish that could not be identified as either male or female by histological examination were analyzed for the genetic sex marker. Genomic DNA was extracted using DNeasy Kit (Qiagen, Germany) from the fin tissue samples that had been stored at –80°C. In brief, the tissue was placed in buffer and homogenized by hand using a sterile, disposable pestle. It was then incubated with 40 μl of the enzyme Proteinase K in a water bath for 1-2 hours at 56°C, and the DNA was extracted and purified through several collection and wash steps according to the kit instructions. Once extracted, all DNA samples were measured for concentration and purity by spectrophotometry (Nanodrop, Fisher Scientific Ltd.). Aliquots of 100 ng DNA/μl were prepared in sterile water for each sample, and stored at -20°C.

The PCR protocol used primers developed by Peichel *et al.* (2004) and modified by Dr. Ioanna Katsiadaki (personal communication) (forward 5'- GGG ACG AGC AAG ATT TAT TGG –3'; reverse 5' – TAT AGT TAG CCA GGA GAT GG - 3'). These primers target the allozyme, isocitrate dehydrogenase, which exists on both the X and Y chromosomes of the three-spined stickleback. Using gel electrophoresis, one product of approximately 302 base pairs (bp) indicates a female and two products (one of 302 bp and the other of 271 bp) indicate a male (Peichel *et al.* 2004).

For PCR, the Accuprime Taq DNA Polymerase System (Invitrogen Inc.) was used to replicate the sex-marker gene. Each sample was prepared in a sterile thin-walled 50 μl PCR tube on ice according to the following method:

1 μl DNA samples (100 ng/μl) + 1 blank + 1 positive control

+

Prepared as a Master Mix on ice; 24 μl was added to each sample:

2.5 μl 10X Buffer II
0.25 μl Forward Primer (10mM)
0.25 μl Reverse Primer (10mM)
0.5 μl Taq Polymerase
20.5 μl dH$_2$0 (autoclaved)

DNA amplification was conducted on the iCycler (Applied Biosystems Inc.) using the following thermal program:

94°C for 5 minutes
95°C for 30 seconds
56°C for 30 seconds
72°C for 30 seconds — Repeated for 40 cycles
72°C for 10 minutes
4°C for ∞

A 2% agarose gel was prepared by adding 1-2 g of Certified PCR Agarose (Bio-Rad Laboratories Inc.) to a clean Erlenmeyer flask (~400 ml) containing either 50 or 100 ml Tris Borate EDTA (TBE) Buffer prepared by:

Adding 9 parts dH$_2$0 to 1 part autoclaved 10X TBE stock:

- 108 g Tris Base
- 55 g Boric acid
- 9.3 g Na$_2$EDTA.H$_2$0 (pH 8.3)
- made up in 1 L de-ionized water

Once the agarose was added to the TBE, the flask was capped with a wad of clean paper towel to prevent evaporation, and heated in a microwave for ~2 minutes until all of the agarose granules had dissolved. Once the liquid was cool enough to hold, 2.5 or 5 μl ethyl acetate was added to the liquid in a fume hood (final concentration of 0.005%), mixed well, and decanted into a prepared mould. A well-comb was placed on top of the gel, and all bubbles and debris were drawn away from the wells to the bottom. The gel was allowed to cool for approximately 20 minutes.

Once cool, the well-comb was removed and the gel was placed in the electrophoresis chamber and submerged in TBE buffer. The replicated DNA samples were removed from the iCycler and placed on ice. Two microlitres of loading buffer were added to each 25 μl sample, and mixed. Twelve microlitres of each DNA-loading buffer sample were then carefully added to each well, alongside 6 μl of 1 kb DNA ladder (Fermentas Life Sciences Ltd.). The gels were run at 80 Volts for 45-60 minutes. Once finished, a photograph was taken of the gel under UV light. Each sample was identified as either female or male by the presence of one or two bands, respectively (Figure 4.13).

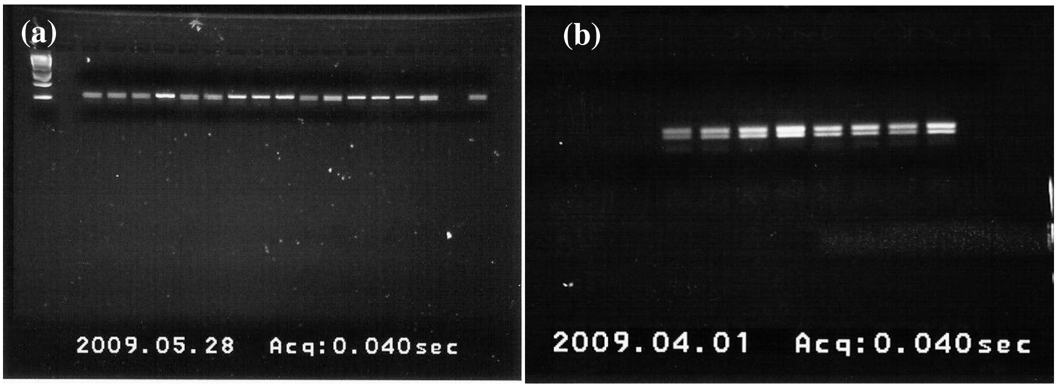
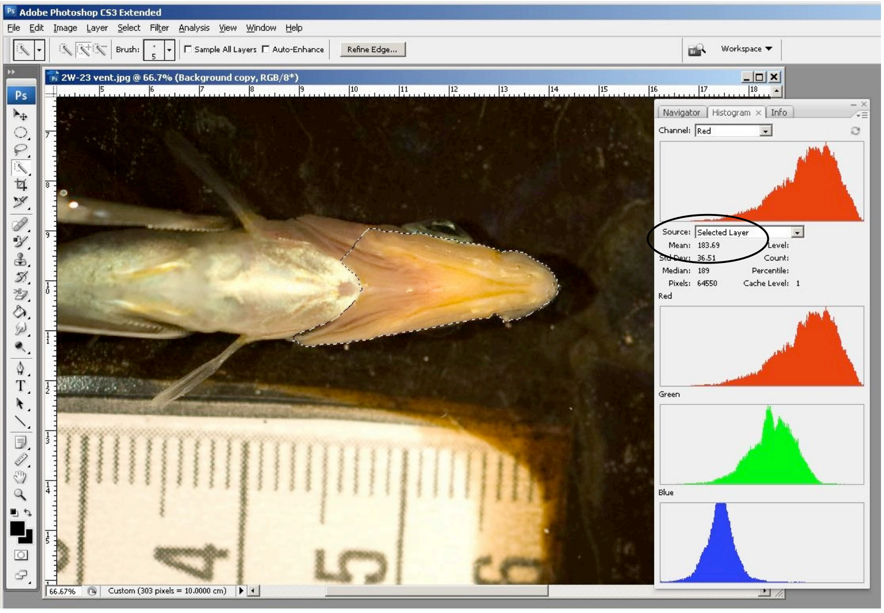


Figure 4.13. Photographs of agarose gels under UV light depicting one or two bands to identify them as female or male, respectively. (a) At a lower magnification, it is not possible to distinguish whether or not there are 1 or 2 bands in the various samples. (b) At higher magnification, these samples can be seen to have two distinct bands, indicating they are all from male three-spined sticklebacks.

4.2.8.3 Digital analysis of red nuptial colouration on the throat

Due to variation in lighting conditions between sampling days, the red nuptial colouration of the males could only be compared within Experiments.



Channel: Red
Source: Selected Layer
Mean: 183.69
Std Dev: 36.51
Median: 189
Pixels: 64550
Level:
Count:
Percentile:
Cache Level: 1

Figure 4.14. Analysis of redness of the nuptial colouration by selection of the area of purest colour (dotted line) and the histogram (Adobe Photoshop CS3) showing the red value for the average colour of this selected area (circled).

The image of the ventral side of each male was analyzed with Adobe Photoshop CS3 Extended (Adobe Systems Ltd.) in which each digital photograph was standardized for size. Using the Magic Selection Tool, the most homogeneous and brightly coloured area of the throat was selected (Figure 4.14). The program then calculated the average colour of the pixels in the selected area, and the red, green, and blue values for that colour. Using these values (R, G, B), the redness of the colour was calculated by:

$$\theta = \arccos \frac{R}{\sqrt{(R^2 + G^2 + B^2)}}$$

In order to understand the calculation, one must understand how a digital colour is calculated by a computer. A digital image is made of millions of tiny dots called pixels. The colour of each pixel (including black and white) is determined by a set of three data values denoting how much red, green, and blue light (each from 0 to 255) make up its colour. In general, the higher the R, G, B values, the lighter the colour, the lower, the darker. If a colour is made up of equal values of two of the three colours these will effectively cancel each other out, leaving the third colour to provide the hue for the pixel. For example R, G, B: 0,0,0 is black, 255, 255, 255 is white, 255, 0, 0 is pure red, and any pixel where G=B is a hue of red. The connection between the digital calculation of a pixel's colour, and how it is perceived by the eye, are depicted by an RGB cube (Figure 4.15). This is in contrast to a spectrophotometer, in which the single value of a wavelength corresponds directly to how we perceive the colour.

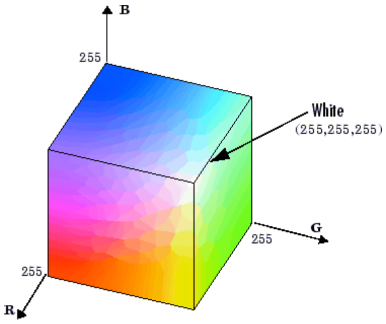


Figure 4.15. An RGB cube depicting how colour histograms relate to how we see colour. Taken from http://www. mathworks.com/ access/helpdesk/ help/ toolbox/ images/f8-15484.html on October 22, 2009.

Several scientists have used a formula of redness, where $r = R/(R+G+B)$, to determine the intensity of red of the throat (Frischnecht 1993). However, this value can only correctly correlate the values of R, G, B to how a colour is perceived within a limited context (ie. when analysing an area of pixels that are generally the same colour and are also a shade of red). If measured outside of this context, a redness value (r) >0.5 is not necessarily red, but instead ranges from dark brown to pale yellow.

The formula developed in the current work tries to account for the length of the vector of that colour within the cube by ($\sqrt{}(R^2 + G^2 + B^2)$), indicating its lightness or darkness, and the angle of the vector from the red axis by θ (theta), indicating the redness of the colour. Generally, as theta approaches 45° it becomes more grey, and if it exceeds 45° it is then no longer red in colour. Therefore the smaller the angle of theta (<45°), the closer the vector of the colour to the red axis, and therefore, the redder the colour. While this formula is unable to represent a colour as seen by the eye as a spectrophotometer would, it does provide a somewhat more accurate index of redness as we perceive it.

4.2.8.4 Spiggin ELISA

Spiggin units/g body weight were determined by Matthew B. Sanders in the laboratory of Dr. Ioanna Katsiadaki at CEFAS, Weymouth, UK according to the methods described in Katsiadaki *et al.* (2002).

4.2.8.5 Kidney cell height measurement in Experiment 2 males

The measurement of kidney epithelium cell height is considered a highly sensitive biomarker correlating closely to spiggin concentrations (Katsiadaki *et al.* 2002). Using the histological slides of the Experiment 2 males in the spawning experiment, a digital photograph was taken of a cross-section of each male kidney with a compound light microscope around the posterior third of the fish, where the gonads generally lie (Figure 4.16a and b). Measurement was conducted using a ruler tool in Adobe Photoshop CS3 Extended (Adobe Systems Inc.).

In order to measure kidney cell height in an unbiased manner, each sample was measured blindly, with no knowledge of which treatment group the sample belonged to. Analysis of the cell heights was also randomized. Each photograph was divided into 80 squares and numbered 1 to 80 from left to right, top to bottom. A table of random numbers was generated, indicating ten randomly selected squares in which the height of the kidney cell was to be measured. To measure the height of the epithelium, the calibrated "ruler tool" was extended from the exterior border of the cell, perpendicularly toward the border of the lumen (Figure 4.16c). Each measurement was recorded, and all measurements were analyzed by one-way ANOVA.

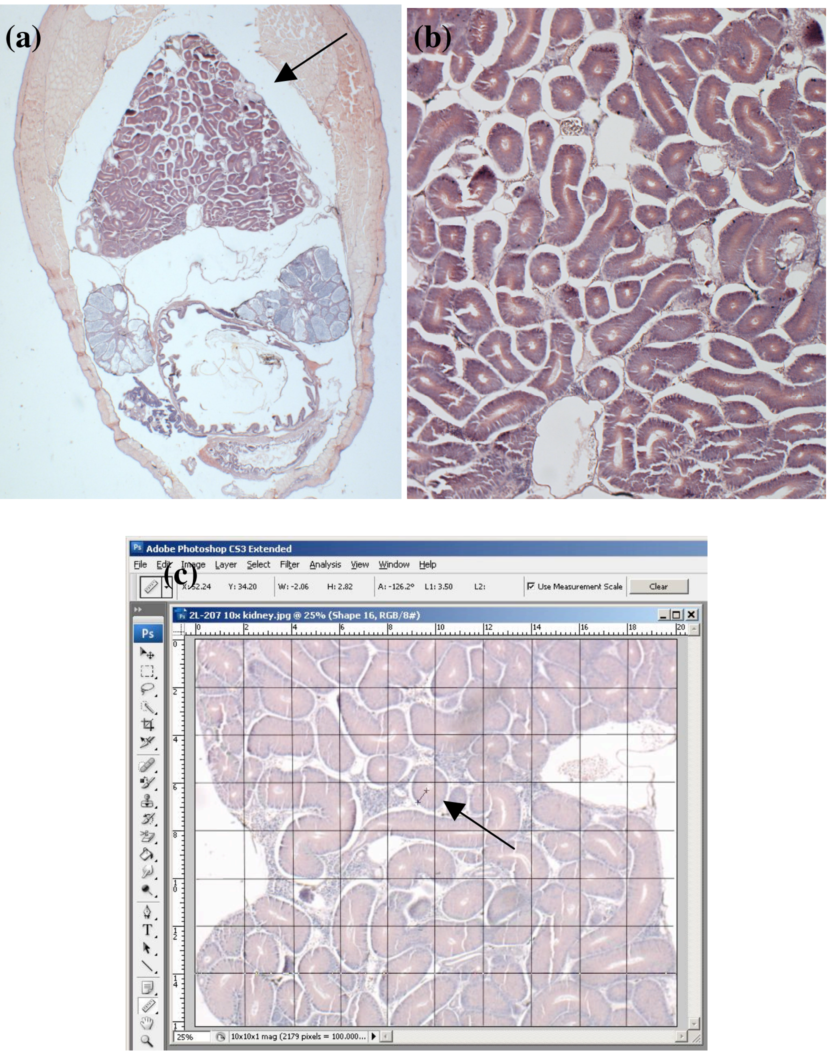


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Figure 4.16. Photographs of a male three-spined stickleback whole-body cross section (a) magnified at 20x (the kidney is indicated by an arrow), (b) with the kidney tissue magnified to 100x, and (c) with the photograph of the kidney in the Adobe Photoshop program divided into an 80-square grid, showing the manner in which the cell height was measured (arrow).

4.2.8.6 Radioimmunoassay of plasma sex steroid concentrations

Radioimmunoassays were conducted following the methods outlined in Chapter 2 (Section 2.2.8). Due to the small volumes of plasma that could be obtained from the fish sampled at 130 dph and 180 dph, only 11-KT concentrations in males were measured at these times. In the nesting and spawning male sticklebacks, concentrations of both testosterone and 11-KT were measured from plasma samples. Conversely, the concentration of 17β-oestradiol (E2) in plasma was measured in the female sticklebacks that had been placed in the nesting and/or spawning from Experiments 1 through 3.

4.2.8.7 *Statistical analysis*

Sigma Stat (version 3.5, Systat Software Inc., Germany) was used to conduct all statistical analyses. The data for plasma hormone concentrations, lengths, weights, gonadosomatic indices, kidney cell height, and the redness of the nuptial colouration and spiggin concentrations were analysed with one-way ANOVA unless they were not normally distributed. In these cases, Kruskal-Wallis ANOVA on Ranks was used with Dunn's Test to determine significant differences. The pooled DBP-exposed 100 μg DBP/L—1 and 2 groups, were analysed by t-test, when compared to the Solvent Control group for lengths and weights. The data for the timing of nest building was analysed by Kaplan-Meier Survival Analysis. The Jonckheere-Terpstra Test was used to compare differences in variation in hormone concentrations between treatment groups Correlations between survival and DBP-exposure, and between spiggin, plasma hormone concentrations, and nuptial colouration were analysed by Pearson Product Moment Correlation. Statistical significance was set at a level of α=0.05.

4.3 Results

4.3.1 Analytical chemistry

The water samples from each experimental group of fish (Experiments 1-3) were measured within 1 or 2 runs on the GCMS. There was no detection of any of the phthalates DEP, DMP, or DnOP in the water samples. However, DEHP was detected in some of the samples and is noted below.

Water samples from Experiment 1 were measured over the course of two runs on the GCMS (September 4th and 19th, 2008). On these days the coefficients of variation of the repeated standards mainly fell between 14 and 20%. However, the coefficients of variation of some of the standard concentrations reached up to 36%, on September 4th, 2008 (for the 2.5 and 5 mg/L concentrations of both DBP and BBP). On September 19th, 2008 the coefficients of variation exceeded 20% for the 20 mg/L standard concentrations (DBP and BBP), and for the 40 mg/L BBP standard concentration. The coefficient of determination was very high for all standard curves ($R^2>0.99$). When DEHP was detected in the Experiment 1 water samples, the mean concentration was 2.25 ± 0.18 μg/L (SD) with 34% incidence overall.

The results of the amassed data for the DBP concentrations in the tanks of the Experiment 1 were very encouraging (Figure 4.17). The Solvent Control tank samples all had a background DBP contamination of 1.96 ± 0.46 μg DBP/L (mean ± SD). The 50 μg DBP/L tank had a measured concentration of 52.28 ± 10.20 μg DBP/L (mean ± SD) throughout the exposure period. Finally, both 100 μg DBP/L—1 and 2 tanks had measured concentrations of 93.68 ± 24.97 and 99.74 ± 17.28 μg/L (mean ± SD), respectively. Therefore, the DBP concentrations measured in the Experiment 1 tanks appeared to accurately represent nominal values.

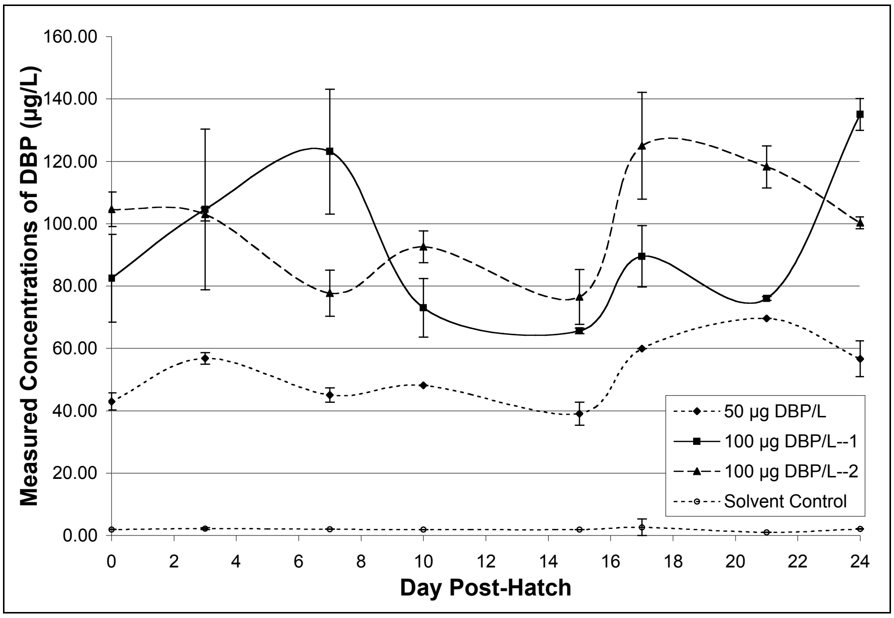


Figure 4.17. Measured concentrations of DBP (mean ± SD) in the fish tanks during Experiment 1, calculated based on the percent recovery of the BBP internal standard, during the 24-day exposure period.

The concentrations of DBP in the Experiment 2 and 3 treatment tanks were measured during two GCMS runs; on September 29th for DBP-treated samples, and on October 13th, 2008, for the Solvent Control samples. During these runs the GCMS coefficients of variation for repeated standards were only 3-18%, and all DBP and BBP standard curves reported coefficients of determination of $R^2 > 0.99$, suggesting both a high degree of precision and accuracy. There was a very low incidence (6%) of DEHP detected in the October 13th, 2008 run only. The average concentration of these DEHP-positive samples was 1.69 ± 0.04 μg/L (SD).

The concentrations of DBP in the tanks during Experiment 2 were also reasonably close to their nominal values. The Solvent Control tank had a mean (±SD) concentration of 2.31 ± 0.55 μg DBP/L with a 100% incidence. The Low Dose tank, with a nominal concentration of 50 μg DBP/L, was more variable, with a mean (±SD) concentration of 74.32 ± 25.68 μg DBP/L, and the mean concentration in the High Dose tank (100 μg DBP/L) was even more variable, at 170.89 ± 49.53 μg DBP/L (Figure 4.18). For an unknown reason, both the 50 and 100 μg DBP/L tanks were measured to have increased concentrations of DBP on Day 20, which skewed the mean values somewhat. Overall, the measured DBP concentrations were slightly above their nominal values.

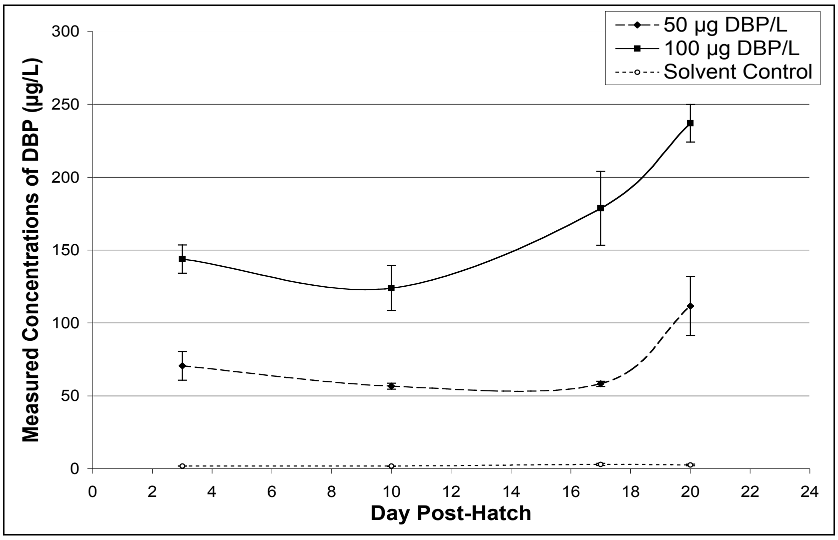


Figure 4.18. Concentrations of DBP (mean ± SD) in the water of Experiment 2 treatment tanks, recalculated based on percent recovery of the BBP internal standard during the 0-24 dph exposure period.

The water chemistry for Experiment 3 yielded somewhat more mixed results than those of Experiments 1 and 2 (Figure 4.19). The Solvent Control tank had very low background DBP concentrations in all samples of 2.32 ± 0.68 μg DBP/L (mean ± SD)). One sample of the Solvent Control tank was measured at 9.38 μg DBP/L, with a recovery of 97% of the internal standard, on Day 23 of the experiment, while its duplicate sample was measured to have a concentration of 0.98 μg DBP/L with 87% recovery of the internal standard. This former concentration was omitted from the data, as it was more than 2 standard deviations from the overall mean DBP concentration of the water samples from the Solvent Control tank. It was likely the result of sample contamination or mix-up, since no other samples in any Solvent Control tanks reported concentrations exceeding 3.5 μg DBP/L. The 100 μg DBP/L tank in Experiment 3 had a measured concentration of 137.87 ± 55.38 μg DBP/L (mean ± SD). Unfortunately, concentrations in the 200 μg DBP/L tank were much more variable, with a measured mean concentration of 467.42 ± 206.33 μg DBP/L (mean ± SD). This was mainly a consequence of the samples collected on Day 13. On this day, the duplicated samples from this tank were measured at 28.33 and 1097.40 μg DBP/L each. This discrepancy was not a result of poor extraction, as both samples recovered >88% of the internal standard. It is possible that the 1097.40 μg DBP/L sample was the result of having collected water from the surface of the water, where accumulated DBP was floating. However, it is unclear why the other sample was measured with such a low concentration of DBP. To ensure this was not a GCMS error, these sample were measured again on October 13th, 2008, and results were similar. Therefore, it remains unclear as to why these duplicate samples were so different.

Overall, the DBP concentrations from Experiment 3 were less uniform than those from Experiments 1 and 2. Thus, it can be said that the Experiment 3 tank with the nominal concentration of 200 μg DBP/L was rather, ">200 μg DBP/L", but how much greater is unknown.

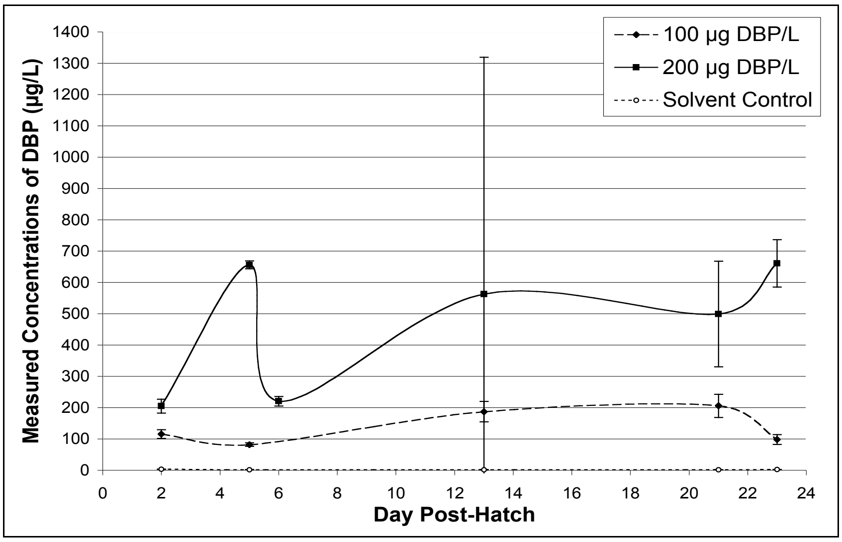


Figure 4.19. The concentrations of DBP in water (mean ± SD) sampled from the Experiment 3 tanks during the 24-day period exposing three-spined sticklebacks to various concentrations of DBP. The concentrations were calculated based on the percent recovery of the BBP internal standard in each sample.

4.3.2 Survival, sex ratio, and growth

4.3.2.1 Survival

Survival could only be assessed in Experiments 2 and 3, since the exact number of eggs added to most of the Experiment 1 tanks was unknown (Table 4.3). Only four fish were present in the 50 µg DBP/L tank in Experiment 1 on October 27, 2008, suggesting a high mortality in this tank. This was did not appear to be related to exposure to DBP, since 100% of the fry in the 100 µg DBP/L –2 tank survived. Further, DBP exposure was not significantly correlated to survival in either Experiment 2 or 3 (P>0.05, df.1, Pearson Product Moment Correlations).

Survival in Experiments 2 and 3 was over 85% in all of the treatment tanks except for the Experiment 3 Solvent Control tank (Table 4.3). On September 16, 2008 (16 dph), several fry were found dead when this tank was cleaned. These fry appeared to be less than 1 cm in length, and were thought to have died around 7 dph. Overall, survival did not appear to be correlated to phthalate exposure.

4.3.2.2 Sex ratio

The male to female sex ratio varied between 0.6 and 1.5 in the Solvent Control groups of Experiments 1 through 3. The sex ratios in the fish tanks treated with DBP varied in a similar manner to those of the controls (Table 4.3).

Table 4.3. Number of three-spined sticklebacks, percent survival, and sex ratios in each treatment tank from Experiments 1, 2, and 3.

Experiment	Treatment	Original Number of Eggs	Number on October 27th, 2008	Survival (%)	Sex Ratios (M/F)
1	Solvent Control	Unknown	48		1.5
	50 μg DBP/L	Unknown	4		N/A
	100 μg DBP/L—1	Unknown	48		1.1
	100 μg DBP/L—2	20	20		0.8
2	Solvent Control	62	62	100	1.0
	50 μg DBP/L	62	62	100	0.6
	100 μg DBP/L	61	52	85.25	1.4
3	Solvent Control	45	16	35.56	0.6
	100 μg DBP/L	46	40	86.96	1.7
	>200 μg DBP/L	45	39	86.67	0.9

4.3.2.3 Growth

Length and weight data were analysed individually and separately in males and females. In both males and females, length and weight of the fish were not significantly different between the Solvent Control tanks and DBP-treated tanks with some concentration-independent exceptions ($P>0.05$, ANOVA).

4.3.3 Results from early sampling (~130 dph)

The three-spined sticklebacks sampled at ~130 dph were analyzed for the histology of the ovaries and testes, and plasma 11-KT concentrations measured in the male fish only. The sample sizes for histological analysis of Experiment 1 Solvent Control and 100 μg DBP/L—1 were n=3 and n=7 males, and n=4 and n=1 females, respectively. Experiment 2 sample sizes for histology were n=7, n=2, and n=8 males, and n=5, n=10, and n=4 females for the Solvent Control, 50 and 100 μg DBP/L tanks, respectively.

The males in all of the treatment groups had Juvenile-stage testes with only spermatogonia present. One male from the Experiment 1 100 μg DBP/L—1 group was noted to have one testis much larger than the other, but this was difficult to confirm in cross section. No abnormalities such as dysgenetic cyst structure or multinucleated gonocytes were noted in the testes, but at such an early stage of sexual development such structures can be difficult to discern (Figure 4.20).

The females had all reached Stage 1 in development: most oocytes were pre-vitellogenic, with some cortical alveolar oocytes present. The ovarian structure appeared normal, with a columnar ovarian cavity wall. There was no evidence of increased oocyte atresia, cell hyperplasia, or accumulated egg debris (Figure 4.21).

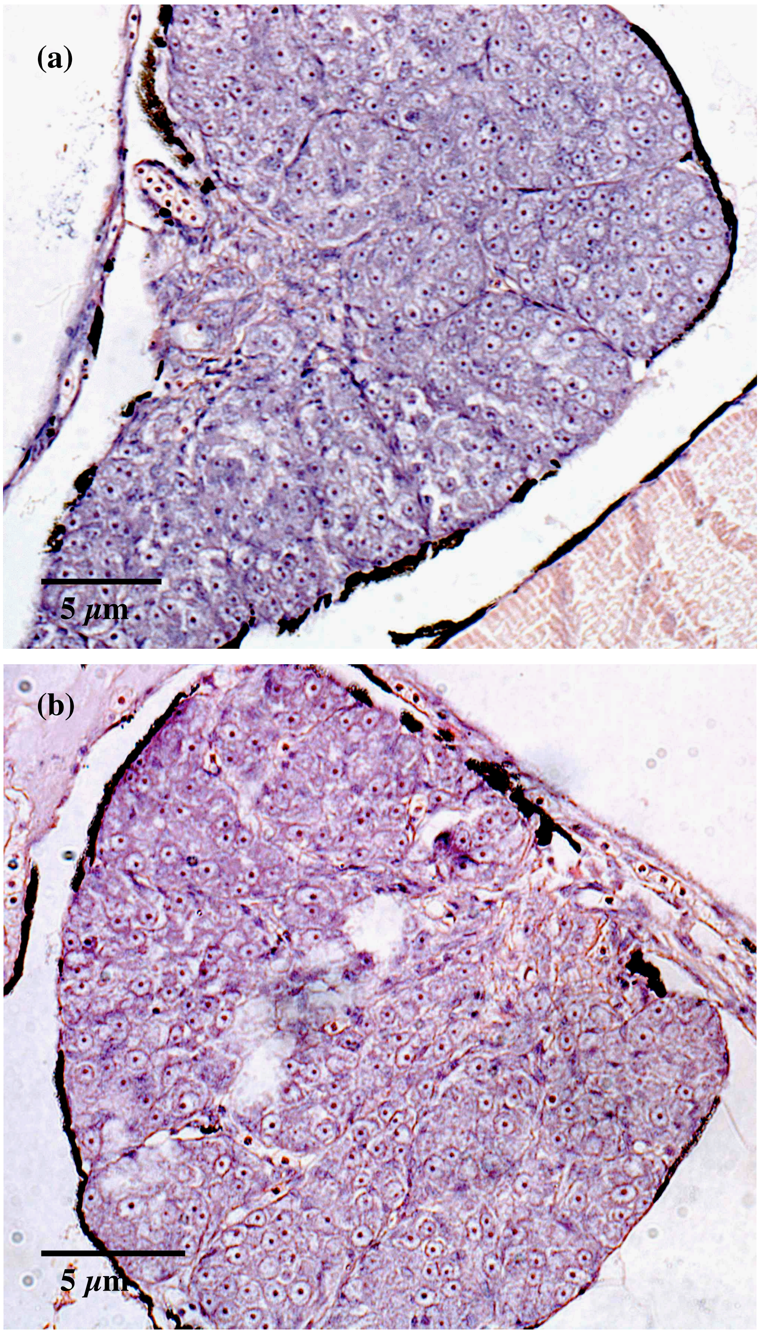


Figure 4.20. Transverse sections of two Juvenile-stage testes (spermatogonia only) from 130 dph-old male three-spined sticklebacks from Experiment 2. These fish were exposed from 0 to 24 dph to DBP and were sampled from (a) the Solvent Control group, and (b) the 50 μg DBP/L group. (Magnification 400x).

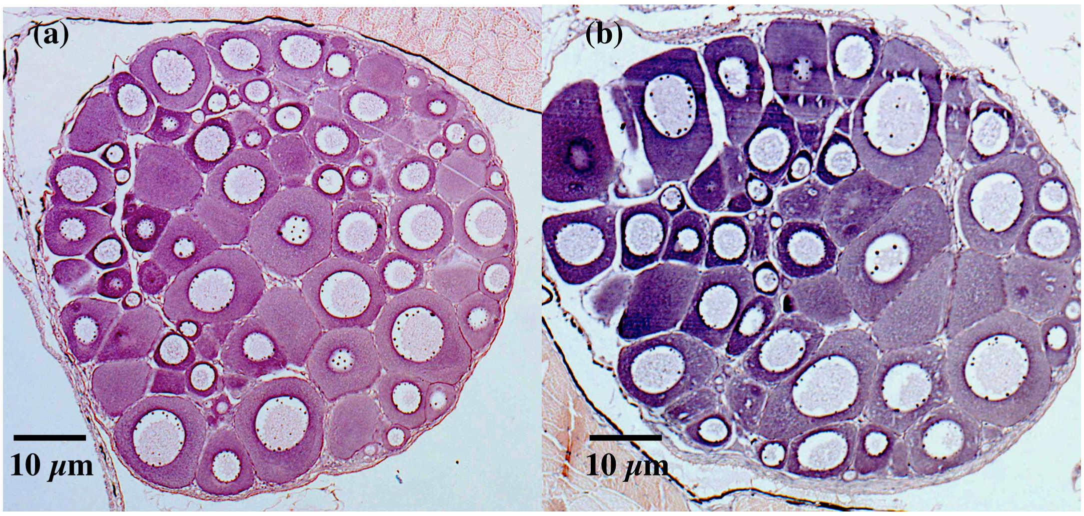


Figure 4.21. Transverse sections of Stage 1 ovaries, containing mostly pre-vitellogenic oocytes, in 130 dph-old female three-spined sticklebacks from Experiment 2 in (a) the Solvent Control group, and (b) the 100 μg DBP/L group. The fish were exposed to DBP from 0 to 24 dph. (Magnification 100x).

Due to the small blood sample volumes, the results for the plasma 11-KT concentration from the male sticklebacks were mainly extrapolated, as their concentrations were too low to fall on the linear part of the standard curve. This probably makes the 11-KT concentrations less accurate than is desirable. The plasma 11-KT concentrations in males were significantly higher in the Experiment 1 100 μg DBP/L –1 group compared to the Solvent Control ($P<0.05$, df. 2, ANOVA on Ranks) (Figure 4.22a). In contrast, in Experiment 2, the fish from the 100 μg DBP/L group seemed to have slightly lower concentrations of 11-KT in their plasma, but this was not observed in the fish from the 50 μg DBP/L group, and was also not significant ($P>0.05$, df. 2, ANOVA on Ranks)(Figure 4.22b).

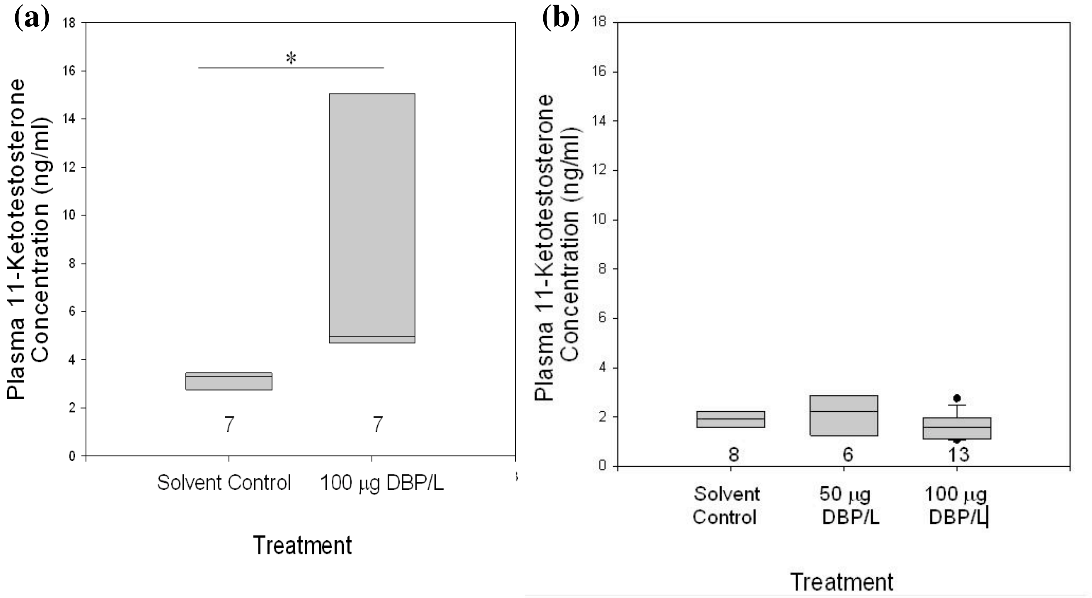


Figure 4.22. Plasma 11-ketotestosterone concentrations measured by RIA in three-spined stickleback males from (a) Experiment 1, and (b) Experiment 2 sampled at ~130 dph following exposure to various concentrations of DBP in early life (*P < 0.05). (Boxes depict the 25th and 75th percentiles about the median, bars extend to the 10 and 90th percentiles, and dots represent outliers. Sample sizes are shown below each box).

4.3.4 Results from late sampling (~ 180 dph)

4.3.4.1 Histology

DBP did not appear to have affected the histological appearance of either the testes or ovaries of the three-spined sticklebacks at 180 dph. Within experiments, males and females in all treatment groups were generally observed to have reached the same gonadal stage. No abnormalities were observed in the testes of the males. In females, the ovaries appeared normal, except for the frequent finding of atretic follicles. These atretic oocytes were generally at a late vitellogenic stage in development and appeared to have disorganized or degraded ooplasm and broken-up chorions (Figure 4.23).

Figure 4.23. Transverse section of an ovary from a female from Experiment 3 that was exposed to a nominal concentration of 100 μg DBP/L in early life. The atretic follicle is marked with an arrow. (Magnification at 100x).

Upon initial examination of the females sampled from Experiment 1, many oocytes in both of the 100 μg DBP/L—1 and 2 groups were noted to have Grade 1-2 severities of oocyte atresia, characterized by "fuzzy" borders on the cells. However, when these samples were re-examined blindly, these cells were concluded not to be atretic because they did not have clumping or perforation of the chorion, fragmentation of the nucleus, disorganized ooplasm, or uptake of yolk granules by perifollicular cells (EPA 2009). Instead, the "fuzzy" cells were determined more to be an artefact of poor histological processing (Figure 4.24).

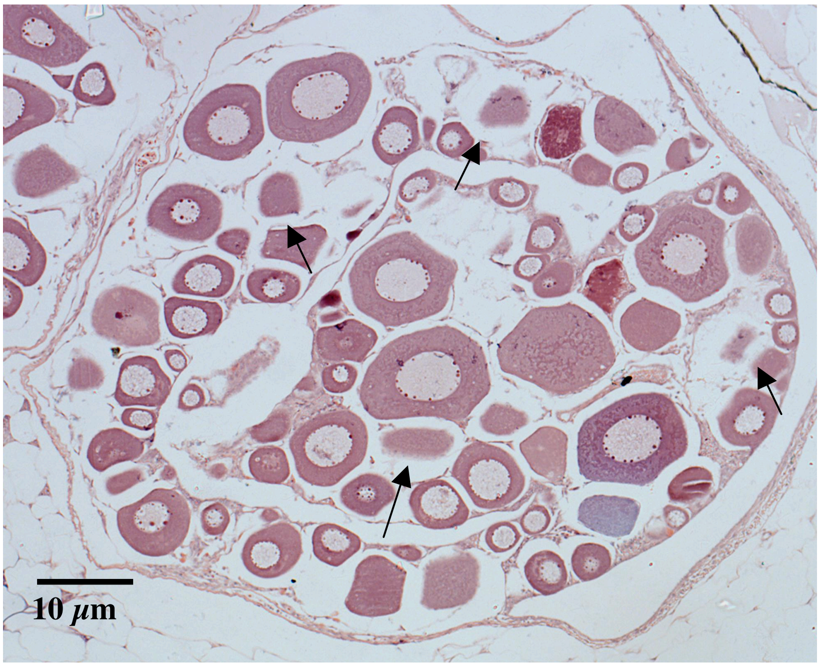


Figure 4.24. A transverse section of the ovary of a female three-spined stickleback from Experiment 1 in the 100 μg DBP/L group at 180 dph. Arrows indicate "fuzzy" cells originally thought to be atretic, but later concluded to be simply an artefact of poor histological processing. (100x magnification).

Again in Experiment 2, all females were at Stage 1, and there appeared to be a high degree of atretic follicles. However, atretic follicles seemed to decrease in frequency with increasing DBP concentration (4 of 4 Solvent Control females, 4 of 6 50 μg DBP/L females, and 4 of 7 100 μg DBP/L females had atretic follicles). The females in Experiment 3 did not appear to have a notable degree of oocyte atresia. It was noted only in one female from the Solvent Control and one female from the 100 μg DBP/L group. This was, however, complicated by the fact that there was only one female sampled from the Solvent Control group. Overall, oocyte atresia did not appear to be clearly linked to phthalate exposure.

4.3.4.2 Plasma hormone concentrations

No clear trends emerged in the analysis of the androgen 11-KT in the plasma of the non-nesting male sticklebacks sampled at 180 dph. In general, the concentration of 11-KT in plasma was measured at roughly the same concentration range in all three Experiments. In several cases, many results were extrapolated, as the concentrations were too low to fall on the linear part of the standard curve. Due to small sample volumes, they could not be reanalysed.

In Experiment 1, treatment of the male fish with DBP from 0 to 24 dph resulted in a significant decrease in plasma 11-KT concentrations in both 100 μg DBP/L tanks compared to the Solvent Control (P=0.002, df. 2, ANOVA on Ranks). This was particularly suprising because of the small sample size in the 100 μg DBP/L—2 group of n=3, which would suggest a high risk of Type I Error. However, when data from the 100 μg DBP/L tanks were pooled together, this difference was even more significant (P<0.001, df. 1, ANOVA on Ranks) (Figure 4.25).

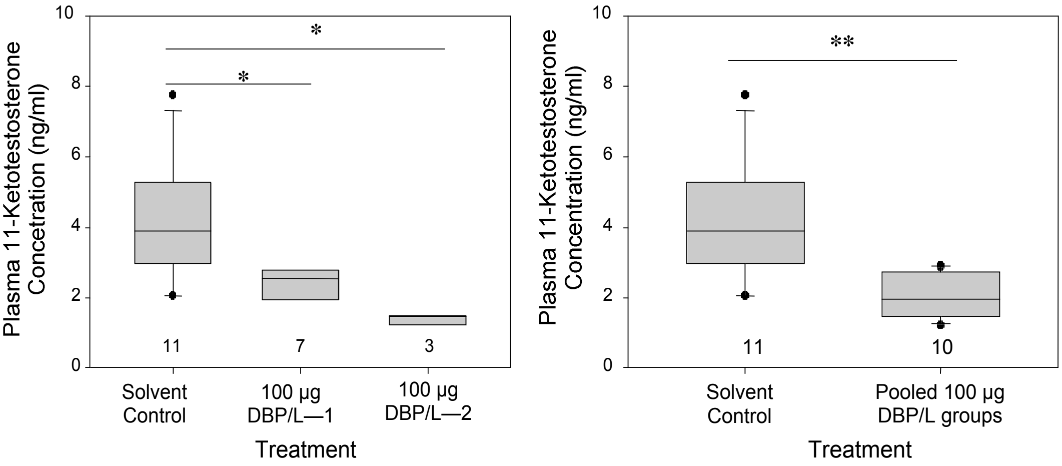


Figure 4.25. Box plots of plasma 11-KT concentrations from 180 dph-old males from Experiment 1 when data is (a) unpooled, and (b) pooled and compared to the Solvent Control. These plasma samples were collected in males prior to the onset of the breeding condition. (*P<0.05, **P<0.001). (See Figure 4.22 for an explanation of how the data are presented.)

By contrast, the results from the males sampled from Experiment 2 showed a non-significant increase in plasma 11-KT concentration positively correlated with increasing DBP concentration (P>0.05, df. 2, ANOVA on Ranks)(Figure 4.26a). This apparent effect was also seen in Experiment 3 males, with non-significantly higher 11-KT concentrations in the >200 μg DBP/L treatment group, but not seen in the fish exposed to 100 μg DBP/L (P>0.05, df. 2, ANOVA on Ranks). However, considering only three males were analyzed from the Solvent Control in this instance, the results should be approached with caution (Figure 4.26b).

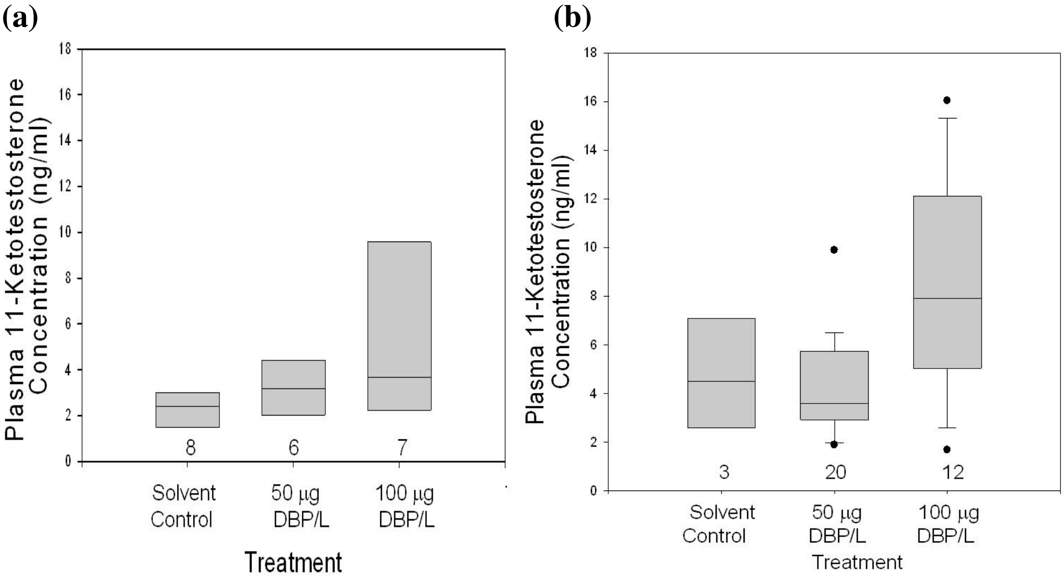


Figure 4.26. Box plots of plasma 11-KT concentrations in male fish sampled from (a) Experiment 2, and (b) Experiment 3. These males at 180 dph had been exposed from 0 to 24 dph to various concentrations of DBP. (See Figure 4.22 for an explanation of how the data are presented.)

Therefore, while the results from the males from Experiment 1 appeared to clearly demonstrate a strong DBP concentration-dependent reduction in plasma 11-KT concentration compared to the control group, the opposite effect was observed in the results from Experiments 2 or 3 at 180 dph, although non-significantly so in these cases.

Interestingly, when the results of the plasma 11-KT concentrations from all three experiments were pooled (effectively pooling only the Solvent Controls and 100 μg DBP/L groups across all experiments), the data seemed to show a different story. In this case, it appeared that there was a DBP concentration-related increase in 11-KT concentration, which was significant for the 200 μg DBP/L group ($P<0.05$, df. 3, ANOVA on Ranks). However, since this group was not actually pooled, we cannot consider this in a direct comparison to the pooled Solvent Control groups. At the lower DBP concentrations, there did not seem to be a particularly strong effect on 11-KT concentration, but the 100 μg DBP/L group did have some higher outliers amongst the results. This suggests that perhaps increased plasma 11-KT concentrations were occurring in some individuals at this DBP-treatment concentration (Figure 4.27).

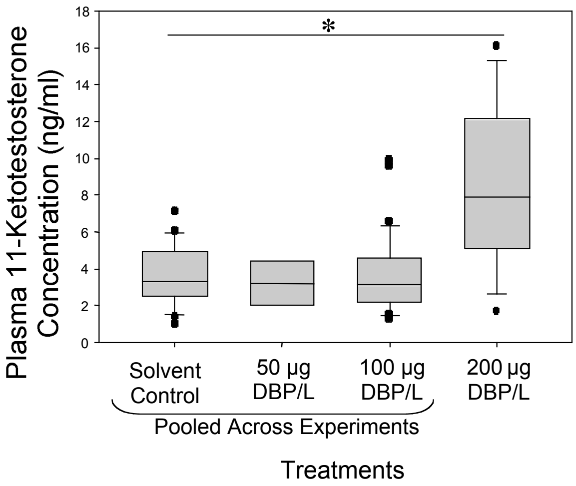


Figure 4.27. Box plot of 11-ketotestosterone concentrations from 180 dph, non-sexually mature three-spined stickleback males in pooled treatment groups from Experiments 1, 2, and 3 that were exposed to DBP from 0-24 dph (*P<0.05; see Figure 4.22 for an explanation of how the data are presented.)

4.3.5 Nesting and spawning experiments

4.3.5.1 Length, weight, and gonadosomatic index

None of the nesting male or female three-spined sticklebacks were observed to have altered lengths, weights or gonadosomatic indices in relation to DBP exposure (P>0.05, ANOVAs).

4.3.5.2 Nest building success and timing

Experiment 1 three-spined sticklebacks were fairly immature at the onset of the nesting experiment. As a result, by the last day of the experiment, the males were not particularly coloured, and only one male in the 100 μg DBP/L—1 group had built a nest. One more male in that same group was considered to have potentially built a small nest. No other nest-building activity was noted in any of the other 18 males.

In Experiment 3, the males were given a longer period in which to build nests. Unfortunately, only three males from the Solvent Control group had been placed in this nesting study, whereas the sample size of the 50 and 100 μg DBP/L groups was n=6 each. While there were no statistically significant trends in nesting behaviour detected with statistical Kaplan-Meier Survival Analysis (P>0.05, df. 2), the males from the Solvent Control group took the longest time to build nests. One male from the 50 μg DBP/L group was the first to build a nest (on Day 20). Two males followed suit on Day 25, one each from the 50 and 100 μg DBP/L groups. Thereafter, the males from the 50 and 100 μg DBP/L groups tended to build nests at a similar rate. However, it took until Day 29 for two males from the Solvent Control to build nests, nine days longer than those exposed to DBP in early life. In the end, 100% of all males from all treatment groups had built nests (Figure 4.28).

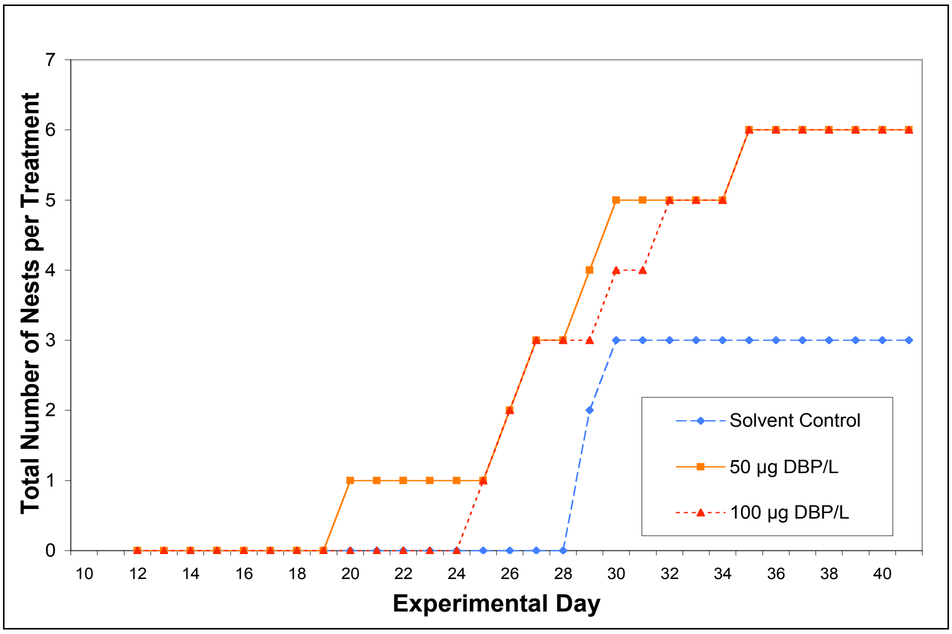


Figure 4.28. The cumulative number of nests per treatment group built over time by male three-spined sticklebacks from Experiment 3 in the 6-week nesting trial, after they had been exposed to various concentrations of DBP from 0 to 24 dph.

The males from Experiment 2 were brightly coloured at the commencement of the nesting trial in March, 2009. Thus, on Day 10 of the experiment, when the nesting material was added, all males began to display nesting behaviours, such as gravel-shifting and the deposition of spiggin on the substrate. By Day 11 100% of males had built nests across all treatments. Once built, these nests were maintained in all of tanks over the course of the spawning experiment. Therefore, there did not appear to be any effect of phthalate exposure on the onset of nest building in Experiment 2 males.

4.3.5.3 Plasma hormone concentrations

The results of the plasma hormone concentrations of fish in each Experimental group are somewhat unique, and depend greatly on the circumstances in which the fish were sampled. This is because the males in each experiment were not sampled at the same age or time following the induction of sexual maturation.

Both plasma 11-KT and testosterone concentrations in the Experiment 1 males were still quite low, because these males had not been given sufficient time to mature. However, the mean concentrations of both hormones in the males seemed to indicate higher plasma androgen concentrations in relation to early life-stage exposure to DBP ($P>0.05$, df. 2, ANOVA on Ranks). Since the fish in the 100 μg DBP/L—1 and 2 groups were exposed to very similar concentrations of DBP, their plasma androgen results were pooled. This resulted in much higher concentrations of plasma androgens in DBP-exposed males, which was statistically significant ($P<0.05$, df. 18, t-test) for 11-KT, but not testosterone concentrations ($P>0.05$, df. 1, ANOVA on Ranks) in plasma, compared to the Solvent Control (Figure 4.29).

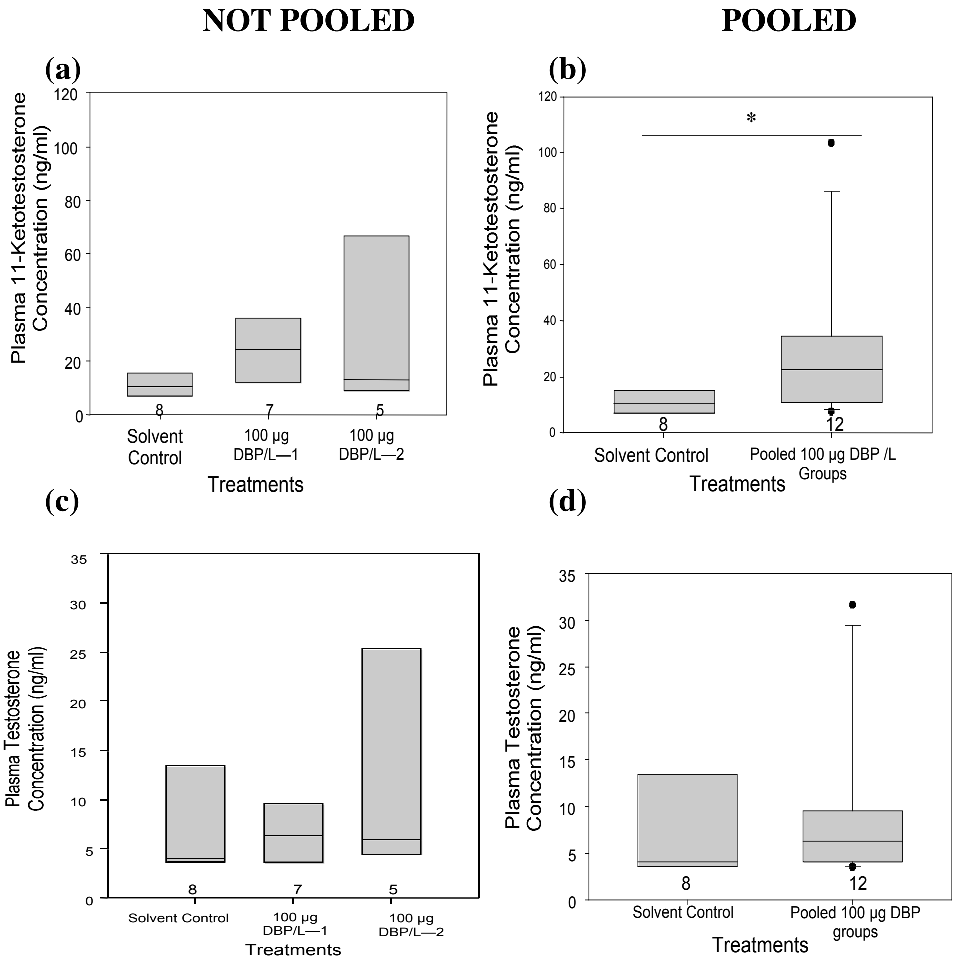


Figure 4.29. Box plots of plasma androgen concentrations from Experiment 1 males sampled after 21 days in a nesting trial. Plasma 11-KT concentrations are compared in (a) unpooled treatment groups, and (b) pooled DBP-treated groups. Plasma testosterone concentrations are compared in (c) unpooled treatment groups, and (d) pooled DBP-treated groups (* P<0.05; see Figure 4.22 for an explanation of how the data are presented).

Females that were placed in individual tanks for the Experiment 1 nesting study (because it was impossible to distinguish between sexes in immature three-spined sticklebacks), were analysed by RIA for concentrations of plasma estradiol. There appeared to be a slight decrease in plasma oestradiol concentration in Experiment 1 females from DBP-treated groups compared to the Solvent Control females, but overall this trend was unclear and non-significant (P=0.092, df. 1 and 2 (in unpooled and pooled groups, respectively), ANOVA on Ranks) (Figure 4.30).

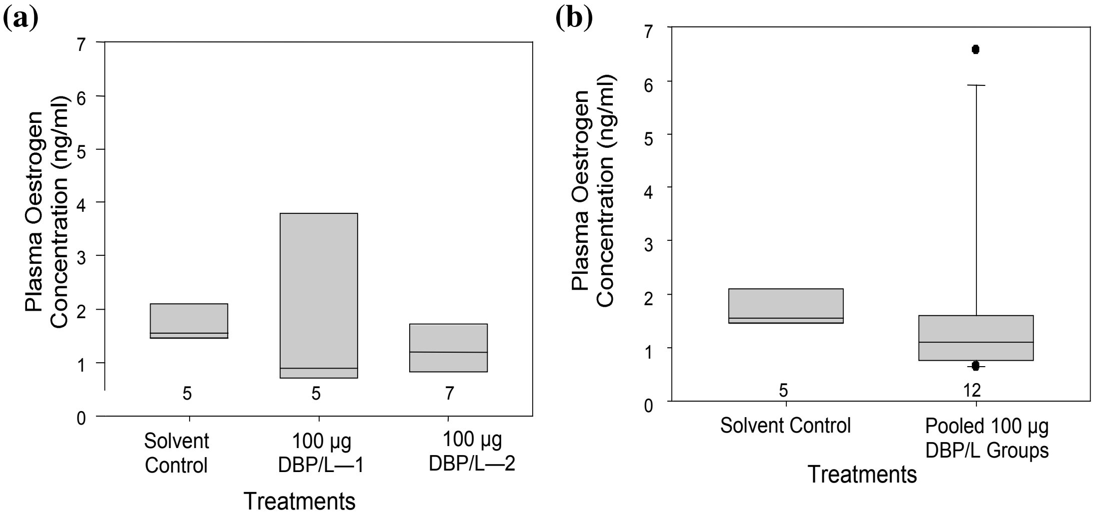


Figure 4.30. Box plots of the plasma 17β-oestradiol concentrations from females from Experiment 1 exposed to various concentrations of DBP following a 21-day nesting study. (See Figure 4.22 for an explanation of how the data are presented).

Experiment 3 males from the nesting experiment were given 6 weeks to build nests and prepare to spawn. This extended time period likely explains why their plasma androgen concentrations were higher than those from Experiment 1. There appeared to be a DBP concentration-related decrease in both plasma 11-KT and testosterone concentrations in this study. This was not significant for either hormone ($P>0.05$, df. 14, ANOVA), but the power of the test for both hormones was lower than desired because of the small sample size in the Solvent Control group (n=3). Thus, false negatives should be considered with caution, especially in the case of plasma 11-KT concentrations, which appear to be DBP concentration-related and have a rather small P-value of 0.069 (df. 14, ANOVA) (Figure 4.31).

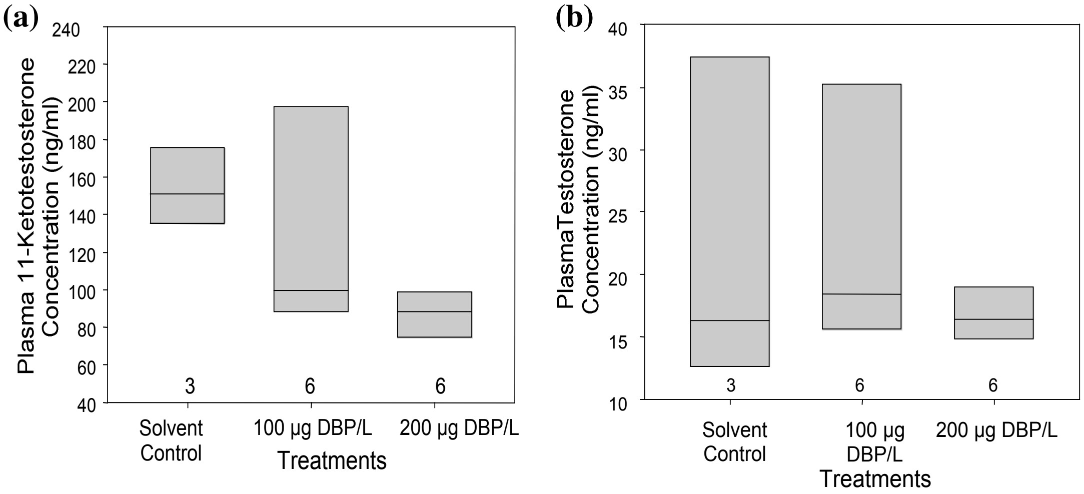


Figure 4.31. Box plots of the concentrations of (a) 11-KT, and (b) testosterone, in the plasma of males from Experiment 3, exposed to various concentrations of DBP from 0 to 24 dph, following a 6-week nesting study. (See Figure 4.22 for an explanation of how the data are presented).

Estradiol concentrations in the plasma of the Experiment 3 females did not appear to be affected by DBP treatment in any dose-related manner compared to the Solvent Control group (P>0.05, df. 20, ANOVA)(Figure 4.32).

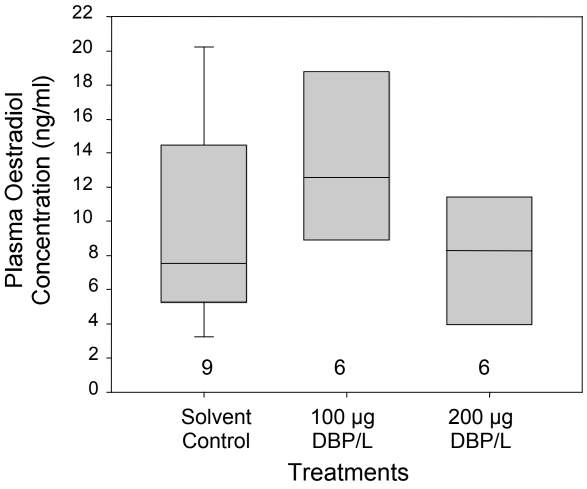


Figure 4.32. Box plot of plasma 17β-oestradiol concentrations from Experiment 3 females, that had been exposed to various concentrations of DBP from 0-24 dph, after a 6-week nesting study. (See Figure 4.22 for an explanation of how the data are presented).

The plasma hormone concentrations of the fish from Experiment 2, that had been placed in the spawning experiment, were analysed on the basis of whether or not the individual fish had spawned. It is well-documented that three-spined stickleback males experience a rapid decline in androgen production immediately after spawning, concomitant with the parental stage of behaviour (Pall *et al.* 2005; Pall *et al.* 2002). Therefore, all fish in the spawning protocol that had spawned were compared only to other fish that had also spawned and fish that had not spawned were also compared to one another.

The mean concentrations of androgens in males that had not spawned did not show a clear trend associated with DBP exposure. Median plasma 11-KT concentrations were higher in DBP-treated males compared to the Solvent Control group, but not in a dose-related manner (P>0.05, df. 9, ANOVA). Plasma testosterone concentrations were slightly higher in the 100 μg DBP/L group compared to the Solvent Control, but the sample size was so small in all groups that this could not be considered related to DBP exposure and was not significant (P>0.05, df. 2, ANOVA on Ranks)(Figure 4.33).

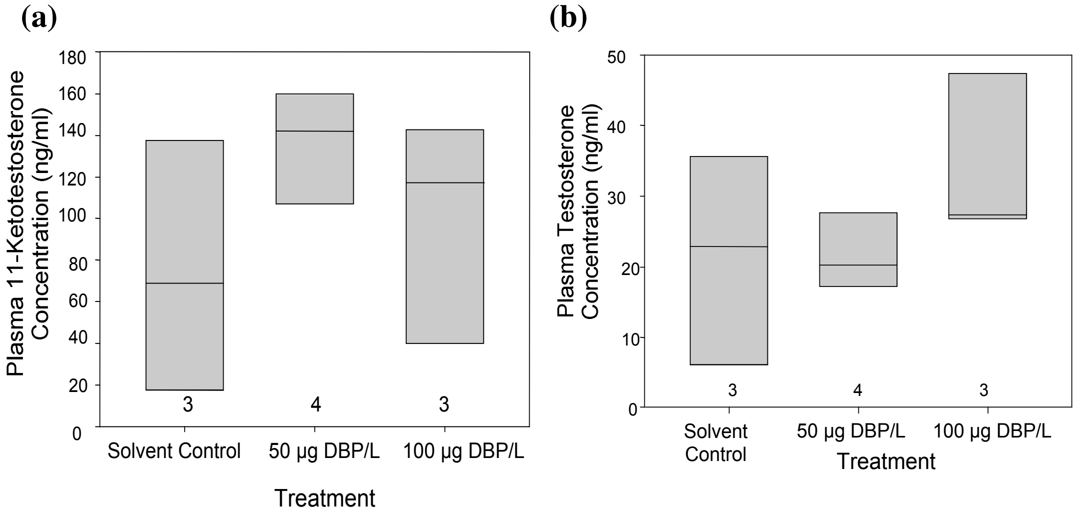


Figure 4.33. Plasma concentrations of (a) 11-KT and (b) testosterone in Experiment 2 males that had not spawned after a 5 week spawning study. (See Figure 4.22 for an explanation of how the data are presented).

In contrast, males that had spawned had much lower plasma androgen concentrations compared to those that had not spawned. Instead of a dose-related increase, there was an inverse correlation between plasma androgen concentrations and DBP concentration in the males that had spawned, but this was not significant for either 11-KT or testosterone concentrations (P=0.182 for 11-KT and P=0.339 for testosterone, df. 9, ANOVA and df. 2, ANOVA on Ranks, respectively). Interestingly, it the 11-KT concentrations did appear to have a significantly different variation with DBP treatment group (P=0.04, Jonckheere-Terpstra Test). However, no trend in variation could be detected for testosterone concentrations (P>0.05, Jonckheere-Terpstra Test). (Figure 4.34).

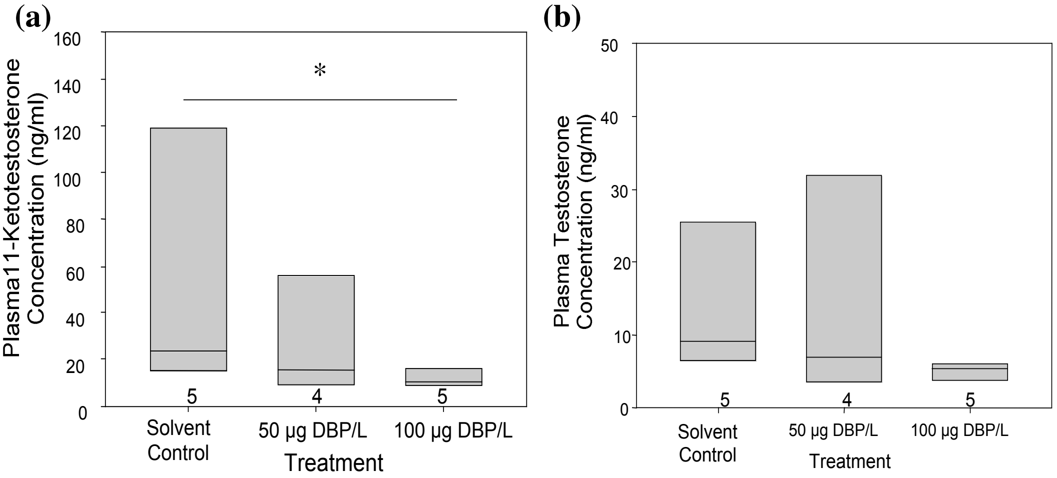


Figure 4.34. Box plots of (a) plasma 11-ketotestosterone and (b) plasma testosterone in spawned males from the Experiment 2 spawning study. (See Figure 4.22 for an explanation of how the data are presented).

Females from Experiment 2 exposed to DBP in early-life that had not spawned did not have markedly different plasma oestradiol concentrations from those of the Solvent Control group (P=0.868, df. 20, ANOVA) (Figure 4.35a). While the group exposed to the highest concentration did have much small variation, there did not seem to be any particular trend related to DBP-exposure for females that had not spawned in the Experiment 2 spawning study (P=0.469, N=14, Jonckheere-Terpstra Test).

The oestradiol concentrations in females that had just spawned exhibited a non-significant positive increase in plasma oestradiol concentration related to DBP concentration. (P>0.05, df. 13, ANOVA) Since the sample size was low, this trend was also not significant (P=0.282, N=14, Jonckheere-Terpstra Test)(Figure 4.35b).

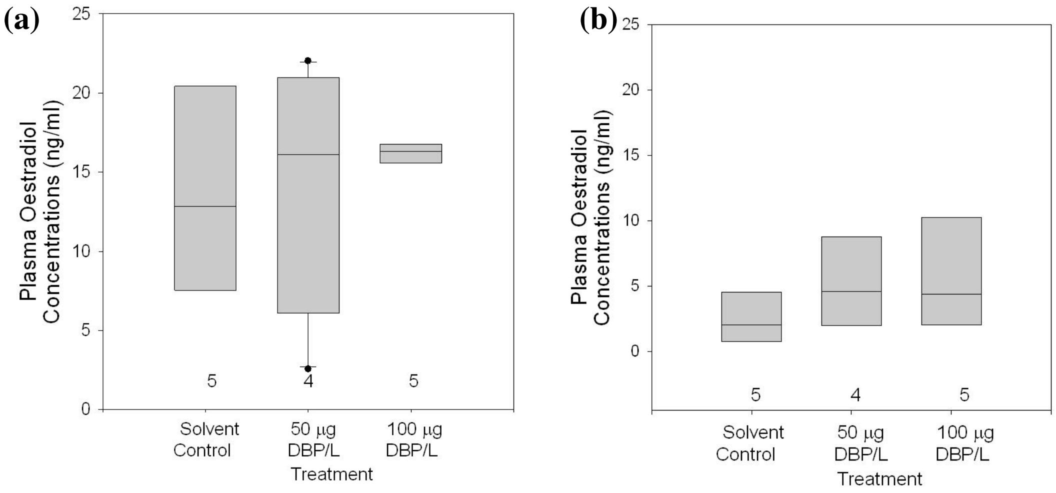


Figure 4.35. Box plots of plasma 17β-oestradiol concentrations in Experiment 2 females that had been exposed to various concentrations of DBP from 0-24 dph, and that either (a) that had not spawned, or (b) had spawned during the spawning protocol. (See Figure 4.22 for an explanation of how the data are presented).

In summary, while DBP did appear to have some concentration-related effects on plasma hormone concentrations, the only significant findings were of increased 11-KT concentration in the plasma of nesting Experiment 1 males, and reduced variation in Experiment 2 males that had spawned. There did not appear to be any discernable effect of early life-exposure of DBP on 17β-oestradiol in mature females. Finally, while no clear trends emerged in any of the nesting and spawning studies, the frequent concentration-related responses suggest that DBP may have been linked to changes in plasma androgen concentrations.

4.3.5.4 Nuptial colouration

The analysis of nuptial colouration was conducted solely from the nesting trials in males from Experiments 2 and 3, because the males from the nesting trial in Experiment 1 were not given enough time to develop nuptial colouration.

In the Experiment 3 males, there was no clear correlation between the redness of the throat based on exposure to increasing DBP concentrations in early life. The mean redness in males from the 100 μg DBP/L group was the highest, while the males from the 200 μg DBP/L group had, on average, the least red throats (P=0.205, df. 14, ANOVA) (Figure 4.36).

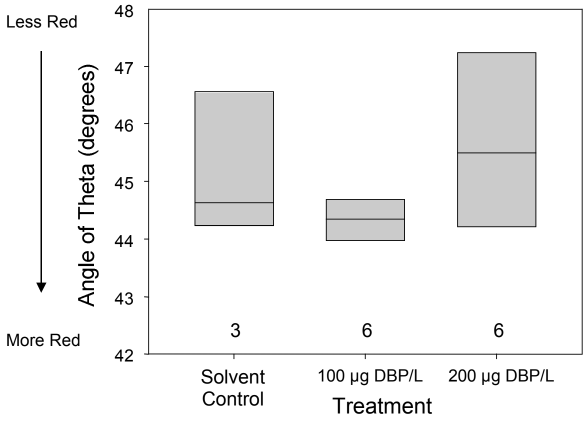
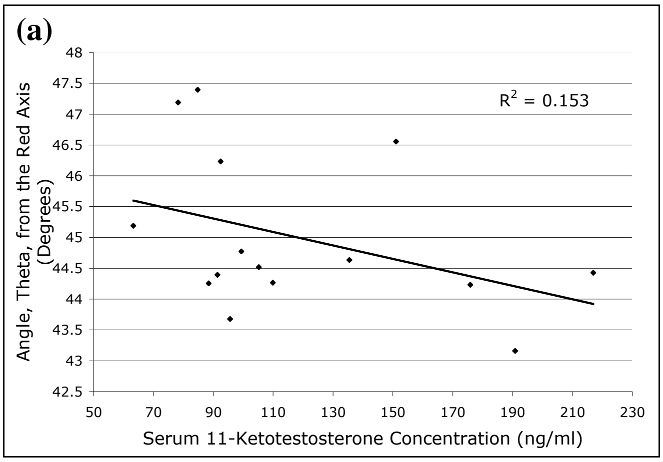


Figure 4.36. Box plot of the angles from the red axis of the throat colours, analyzed from photographs of male three-spined sticklebacks in Experiment 3 in a nesting study for 6 weeks, following exposure to various concentrations of DBP from 0-24 dph. (See Figure 4.22 for an explanation of how the data are presented).

Interestingly, while no relationship could be found between DBP-exposure and nuptial colouration, when plotted against 11-KT or spiggin, the angle of throat redness was negatively correlated with both parameters. The coefficients of determination were weak ($R^2 = 0.153$ for 11-KT; R^2=0.139 for spiggin), but the relationship, in both cases, suggested that the higher both 11-KT and spiggin were, the redder the throat (Figure 4.37). When throat redness plotted against plasma testosterone concentrations, there was little evidence of a correlation between the two (R^2=0.053). Overall, there were no significant correlations between nuptial colouration and spiggin, 11-KT, or testosterone (P>0.05, n=15, Pearson Product Moment Correlation).



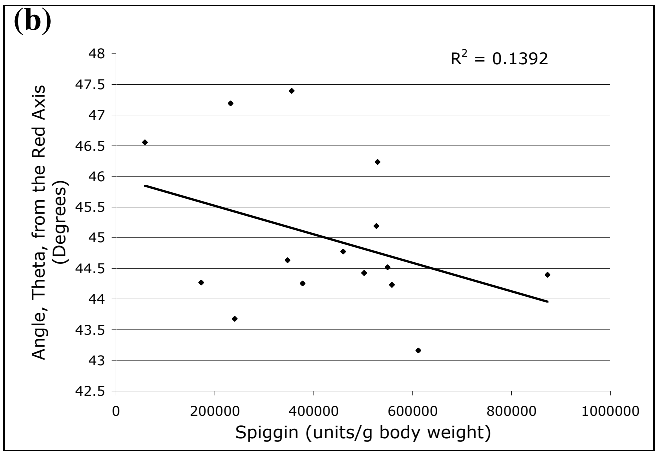


Figure 4.37. Regression correlation between throat redness and concentrations of (a) plasma 11-ketotestosterone, and (b) spiggin, in Experiment 3 males from a 6-week nesting study after early life-stage exposure to various concentrations of DBP from 0-24 dph.

The redness of the throats of males from the Experiment 2 spawning study were also calculated despite the fact that these males had not been photographed under standardized lighting conditions. There was no apparent effect of DBP on nuptial colouration (P=0.550, df. 2, ANOVA on Ranks) (Figure 4.38). When the individual angles for redness of each male throat were plotted against their plasma 11-KT and testosterone concentrations, there appeared to be no correlation between these parameters in Experiment 2 fish (R^2=0.0089 for 11-KT, and 0.0373 for testosterone; P>0.05, n=22, Pearson Product Moment Correlation). This was an even weaker relationship than that of the Experiment 3 fish (Figure 4.37). As a whole, these data suggested that plasma androgen concentrations were not inversely correlated to the redness of the throat in sexually mature male three-spined sticklebacks in the nesting studies.

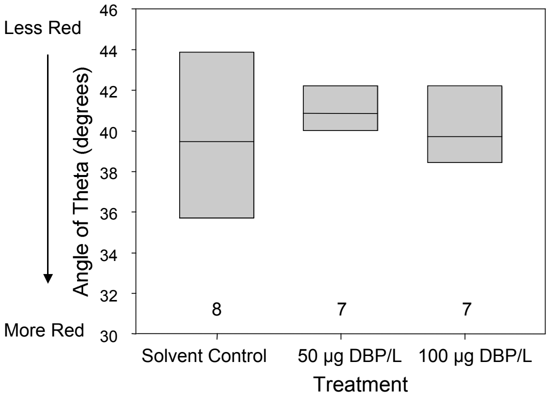


Figure 4.38. Box plot of Experiment 2 male nuptial colouration of the throat, determined by the angle between throat colour and the red axis, in males in groups of fish treated with various concentrations of DBP from 0-24 dph. (See Figure 4.22 for an explanation of how the data are presented).

4.3.5.5 Spiggin and kidney cell height

The spiggin concentrations measured by the CEFAS Laboratory at Weymouth provide several interesting results. In Experiment 1, several males were too small for the kidney sample to be collected. Of those that were analysed, several kidney samples in the Solvent Control group all had spiggin levels too low to quantify on the standard curve, and thus, they were extrapolated. Interestingly, the males that had been exposed in the 100 μg DBP/L—1 and 2 groups had markedly higher spiggin concentrations than Solvent Control males. However, this was not significant even when the spiggin concentrations from the two groups of DBP-treated males were pooled (P=0.200 not pooled, df. 1; P=0.064 when pooled, df. 1, ANOVA on Ranks) (Figure 4.39). When the spiggin concentrations for each individual fish were plotted against their plasma 11-ketotestosterone concentrations, there was a statistically significant correlation (R^2= 0.75) between the two parameters by linear regression (P=0.0002, n=12, Pearson Product Moment Correlation) (Figure 4.41a). It was also possible that increased light intensity in the raised tanks at the rear may have influenced the results (Figure 4.11) since it is a strong influence on the onset of breeding condition. To ensure this light intensity did not bias the results, the maximum light intensity in each tank was also plotted against spiggin concentrations but there appeared to be no correlation between the two parameters (R^2=0.030).

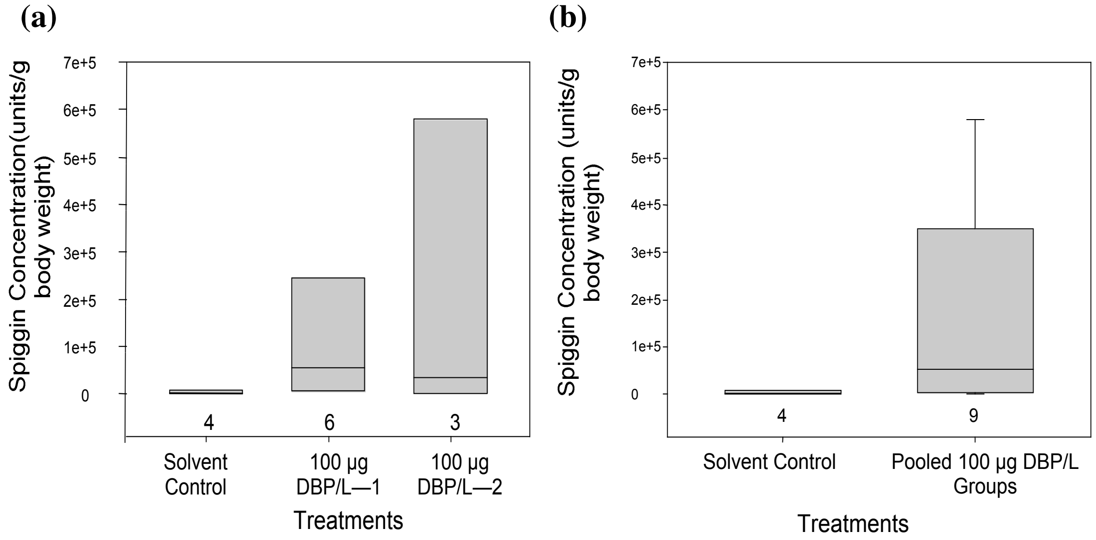


Figure 4.39. Box plots of spiggin concentration in males from Experiment 1 following a 3-week nesting study after having been exposed to DBP during early life-stages with (a) unpooled and (b) pooled 100 *µ*g DBP/L groups. (See Figure 4.22 for an explanation of how the data are presented).

The spiggin concentrations of Experiment 3 males were much different than those of Experiment 1. After the 6-week nesting trial, these males did not appear to differ in their spiggin concentrations based on their treatment group, although the sample size of the Solvent Control was very small (n=3) (P=0.641, df. 14, ANOVA) (Figure 4.40).

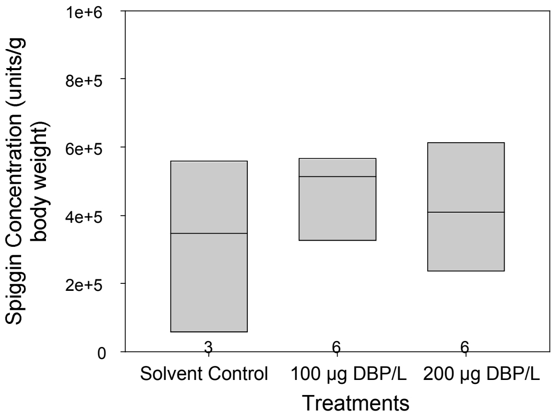


Figure 4.40. Box plot of spiggin units per gram body weight in nesting Experiment 3 males that had been exposed to various concentrations of DBP during early life. (See Figure 4.22 for an explanation of how the data are presented; in this case, sample sizes are shown above each box).

When plotted against plasma 11-ketotestosterone, the results indicated these parameters were not correlated ($R^2 = 0.0067$)($P>0.05$, n=15, Pearson Product Moment Correlation)(Figure 4.41b).

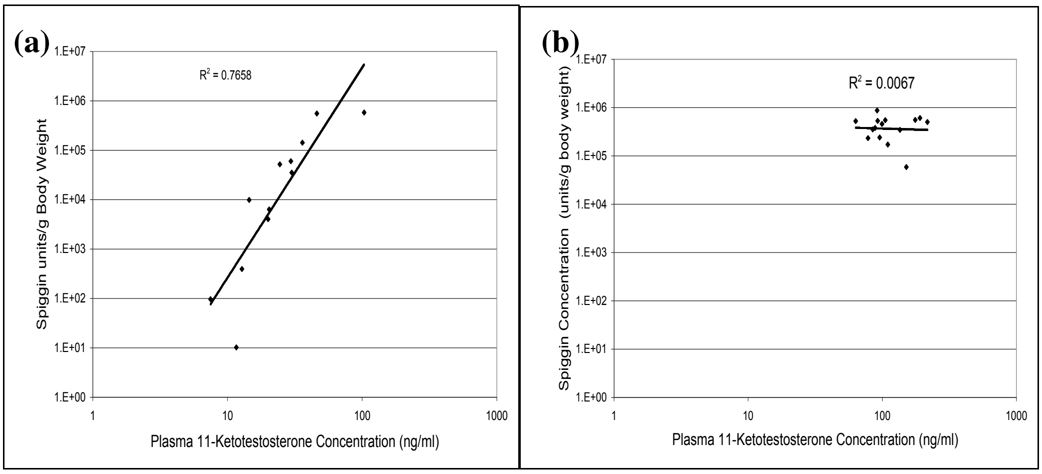


Figure 4.41. Correlation between plasma 11-KT concentration and spiggin concentration in three-spined stickleback males on logarithmic scales from a) Experiment 1 males from the 3-week nesting study, and b) Experiment 3 males after a 6-week nesting experiment, following exposure to various concentrations of DBP from 0-24 dph.

Experiment 2 male kidneys were not removed to measure spiggin concentrations directly. Instead, the spiggin concentrations in these males were measured indirectly by measuring the height of the kidney epithelial cells. There were no discernible differences in mean kidney cell height between the different treatment groups, suggesting spiggin concentrations were not related to DBP-exposure (P=0.732, df. 23, ANOVA) (Figure 4.42). When I compared the cell heights by whether or not each male had spawned, there were still no differences between any of the kidney cell heights of males in the DBP-treated groups compared to those in the Solvent Control group (P=0.762, df. 12 (spawned), df. 9 (not spawned), ANOVA). As in Experiment 3, there also did not appear to be a positive correlation between 11-KT concentrations and kidney cell height in the Experiment 2 males (R^2=0.146, P>0.05, n=24, Pearson Product Moment Correlation).

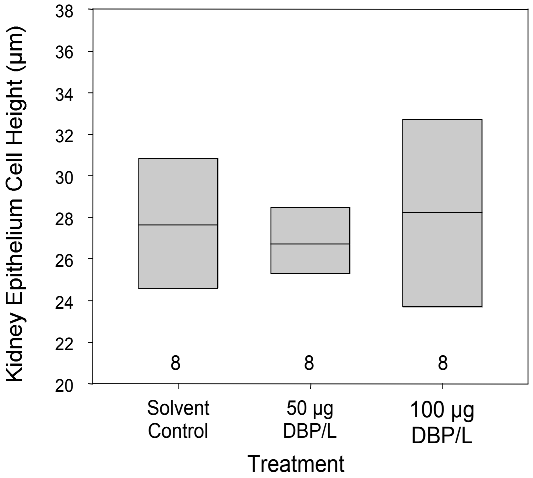


Figure 4.42. Box plot of kidney epithelium height in Experiment 2 male three-spined sticklebacks in different DBP-treatment groups after a 6-week spawning study. (See Figure 4.22 for an explanation of how the data are presented).

In summary, DBP-exposure appeared to increase spiggin concentrations in the Experiment 1 males, but non-significantly. This may be due to the time when the fish were sampled, as they had just begun to produce spiggin, suggesting DBP may alter the timing of the onset of the breeding condition. However the kidney cell heights and spiggin concentrations in the Experiment 2 and 3 males, respectively, did not appear to be altered by early life-stage DBP exposure. This may be because they were sampled after a longer period in the nesting studies (5 and 6 weeks, respectively). In these experiments, it appeared that spiggin was produced in the DBP-exposed groups at concentrations comparable to those in the Solvent Control groups. Thus, it appears that early life-stage exposure to DBP does not prevent three-spined stickleback males from producing similar concentrations of spiggin once they have reached full sexual maturity.

4.3.5.6 Histology of Experiment 2 fish

The gonads of males in the Experiment 2 spawning experiment were mainly found to be in Stage 3 of development, which is defined by the presence of all phases of germ cell division (spermatogonia to spermatozoa), but in which spermatozoa predominated (Figure 4.43). Two males were found to have testes at Stage 2: one from the Solvent Control and the other, from the 100 μg DBP/L—1 group. There was some variation in the thickness of the germinal epithelium in the testes of some males, but when re-analyzed blindly, it did not appear to be related to DBP concentration. No abnormalities, such as intersex, testicular degeneration, or vitellogenin accumulation were noted in any of the histological samples.

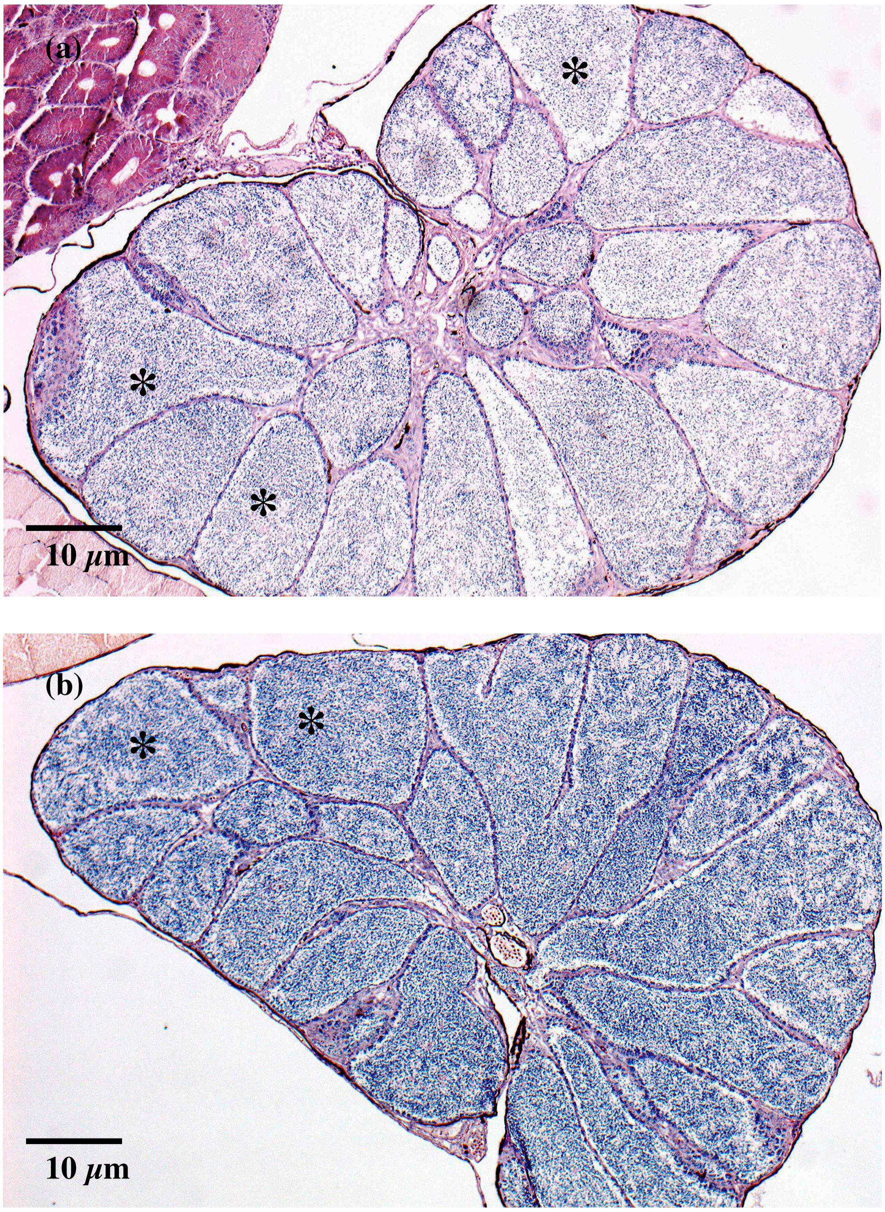


Figure 4.43. Photographs of testes in cross-section at Stage 3 from two sexually mature males from Experiment 2 from the (a) Solvent Control group, (b) 100 μg DBP/L group, following a spawning study, after exposure to DBP from 0-24 dph. (* The large areas filled with dots are examples of lumens filled with sperm).

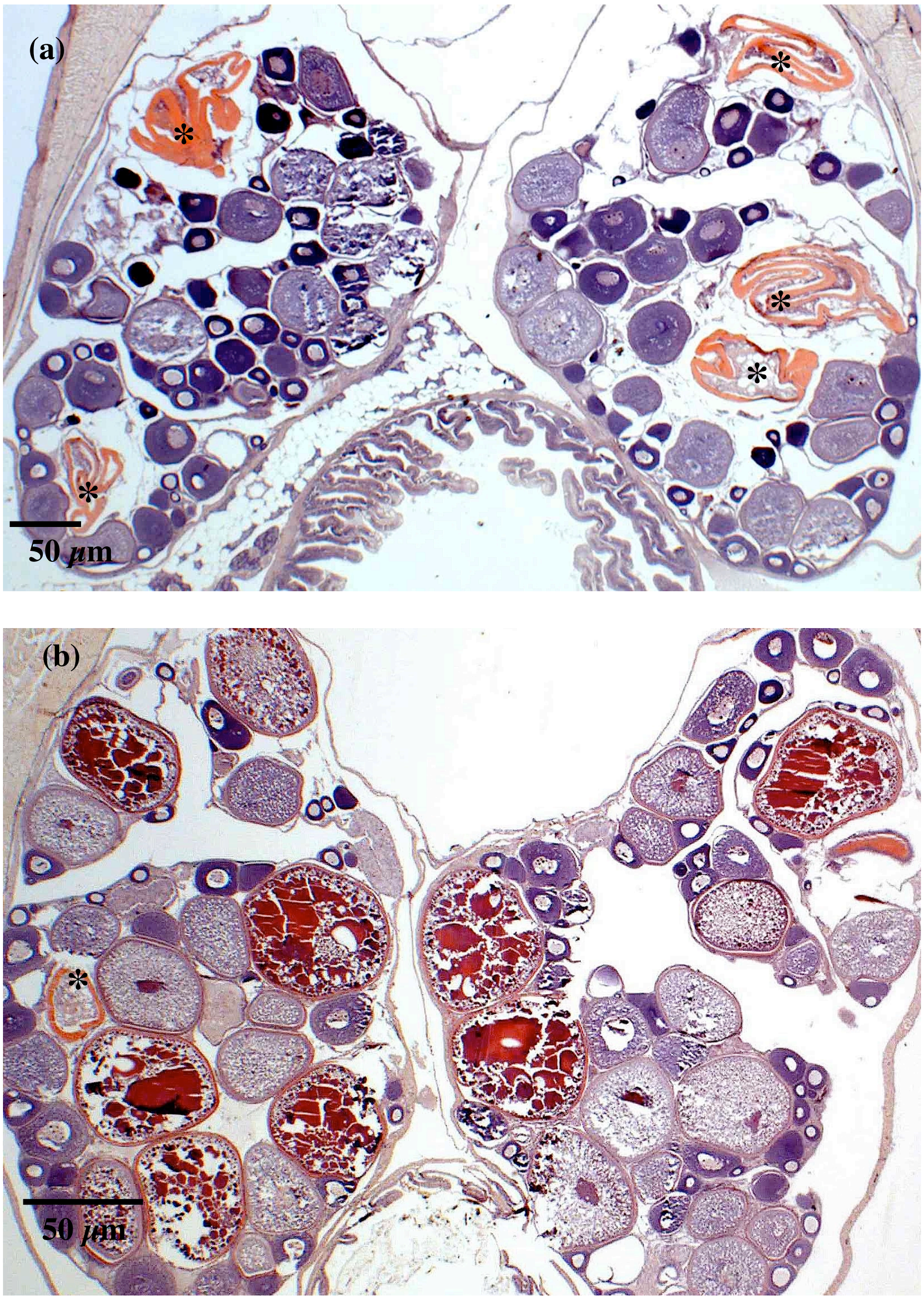


Figure 4.44. Photographs of ovaries in cross-section from Experiment 2 females in (a) the Solvent Control group, and (b) the 100 µg DBP/L group, from a spawning experiment, following exposure to various concentrations of DBP in early life. (*Atretic follicles with infolded chorions).

In the Experiment 2 females across all DBP-treatment groups, the ovaries examined spanned a wide range of gonadal stages, from Stage 1 (ovaries with predominantly pre-vitellogenic oocytes, with some cortical alveolar oocytes present) through to Stage 5 (in which the female is spent and mainly spent follicles remain)

(Figure 4.44). During the initial inspection, several females were noted to have late vitellogenic oocyte atresia. All of the female histological samples were re-analysed blindly in order to determine if this phenomenon was concentration-related and the results are presented below (Table 4.4).

Table 4.4. Distribution of oocyte atresia and its severity (grade) in mature Experiment 2 females from a spawning study that had been exposed to various concentrations of DBP during early life-stage (0-24 dph).

Treatment	Total Number of Females Analysed	Number of Females in which Oocyte Atresia was Observed	Mean Grade of Atresia in Atresia-Positive Females	Range of Grades in Atresia-Positive Females	Fraction of the Total Number of Females with Some Atresia
Solvent Control	13	6	2	1-4	0.46
50 μg DBP/L	14	6	1.16	1-2	0.43
100 μg DBP/L	7	6	1.43	1-3	0.88

Interestingly, there did not appear to be a difference in the severity of oocyte atresia between the Experiment 2 females exposed to DBP and the Solvent Control females. In fact, the highest degree of severity of atresia (Grade 4) was noted in the ovaries of two females from the Solvent Control group. Conversely, there was a much higher proportion of females in the 100 μg DBP/L group who were observed to have some form of atresia, 88% compared to 46% in the Solvent Control, and 43% in the 50 μg DBP/L groups. It should be noted once again, that Dr. Jeffrey Wolf (Dr. of Veterinary Medicine at Experimental Pathology Laboratories in Sterling, Virginia, USA) often observes late vitellogenic oocyte atresia in female fish. Upon inspection of some of these histological samples, he reported that these eggs are mature but have not ovulated and are in the process of being resorbed, stating this occurs commonly in laboratory females when not given an opportunity to spawn. In response, the atresia-positive females were plotted according to whether or not they had spawned. However, there did not appear to be a clear relationship between spawning and the presence or absence of atresia (Figure 4.45).

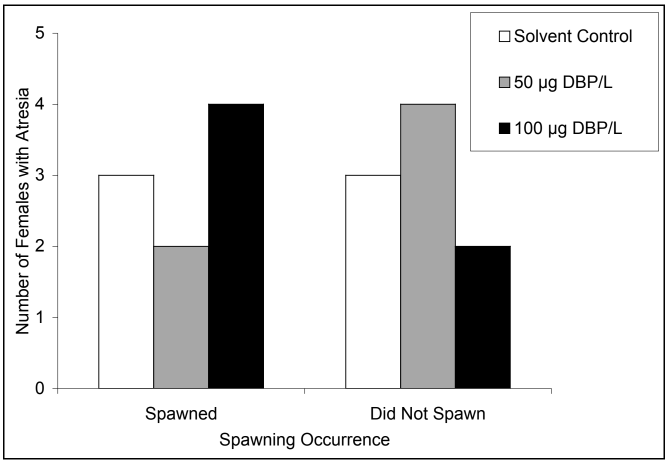


Figure 4.45. Bar graph comparing whether spawning is related to the observation of oocyte atresia in females exposed to DBP during early life-stages.

4.3.5.7 Spawning success

Males from all of the treatment groups in Experiment 2 were able to produce viable fry surviving from at least, 5 to 8 days post-hatch. However, hatching was not observed in some of the nests despite the observation that spawning had occurred. The reason for this was not determined, but possibilities include male cannibalism of the eggs or human error in assessing whether or not spawning had occurred. These hatch-less nests were observed for 8-12 days for any sign of fry hatch, which was three days longer than the 5-9 days normally required for the fry to hatch from most nests (Table 4.5).

Table 4.5. Spawning and hatching success of three-spined stickleback males from the spawning study of Experiment 2, after they had been exposed to various concentrations of DBP during early life.

Treatment	Number of Males That Spawned	Number of Nests Containing Eggs from which Fry Hatched	Percent of Nests Containing Eggs from which Fry Hatched	Total Number of Fry Hatched	Number of Fry per Successful Nest (Mean ± SD)
Solvent Control	5 of 8	3 of 5	60%	146	48.67 ± 11.93
50 μg DBP/L	4 of 8	3 of 4	70%	70	23.33 ± 16.92
100 μg DBP/L	5 of 8	4 of 5	80%	109	27.75 ± 31.63

There are several unknown factors which may have influenced these data:

(i) Whether some fry did hatch from these “unsuccessful” nests but were consumed by the males, unbeknown to us.

(ii) Whether some fry from “successful” nests were also consumed by the males.

(iii) How many eggs from each brood hatched, and how many did not.

(iv) Whether the degree of variation in the number of fry per nest is due to natural variation, or is related to DBP-exposure.

With so many unknown variables, it is impossible to draw any specific conclusions on the fecundity of the fish based on the results presented in Table 4.5. However, it can be said that three-spined sticklebacks exposed to DBP from 0-24 dph successfully produced viable offspring at all exposure concentrations.

4.4 Discussion

Early life exposure to DBP appeared to interfere with the normal endocrine system of the three-spined stickleback, but exactly in what way, is unclear. DBP was able to reach the tanks close to their target concentrations in all of the Experiments, with the exception of the 200 μg DBP/L tank in Experiment 3 (Figures 4.17, 4.18, and 4.19). It is likely that the concentrations in this tank generally exceeded the nominal concentration of 200 μg DBP/L during the majority of the exposure period and therefore represents very high DBP exposure if not 200 μg/L *per se* (Figure 4.19).

The successful exposure of the three-spined sticklebacks to concentrations of DBP close to their nominal concentrations was encouraging, and thus we feel the results presented here are true responses of the three-spined sticklebacks to early life-stage phthalate exposure. However, the main frustration of this work was the small sample sizes in the nesting experiments. These sizes were the result of chance, inexperience, and spatial constraints. Both Experiments 1 and 3 depended on the random selection of three-spined sticklebacks for the experiment and the hope that at least 50% of the fish from each treatment group would be male, for a sample size of at least 6 males. While the number of males in each group were adequate in the Experiment 1 nesting study, due to inexperience its duration was not long enough for the fish to come into breeding condition and build nests. This was adapted for the second nesting study using Experiment 3 fish, but the Solvent Control group only had three males, undermining the results. Finally, while the Experiment 2 nesting study used only males, the low incidence of spawning success meant that the spawned and unspawned males were analysed separately in several cases, leading to sample sizes much smaller than required for proper statistical analysis. Despite this, the fact that we were able to repeat the experiment three times (with some variation), provides some power to our results.

4.4.1 Survival, sex ratio, and growth

In terms of early life-stage effects, DBP was not acutely toxic to the fry and did not appear to affect growth or alter the sex ratio (Table 4.3). It is still unclear why such high mortality occurred in the Experiment 3 Solvent Control tank. This was likely not related to exposure to methanol, which had no effect on the survival of the fish in the other Solvent Control tanks. A previous study exposing Japanese medaka during either the egg-stage or at 14-days after hatch found that nominal concentrations of DEHP of 0.01, 0.1, 1.0 and 10 μg/L also did not affect survival or sex ratios compared to controls. Some groups of fish exposed to DEHP did take significantly longer to hatch, but this was not concentration-dependent (Chikae *et al.* 2004; Chikae 2004).

4.4.2 Effects of DBP on the histology of the gonads

In the fish sampled at 130 dph, no discernible effects of DBP were noted on the histology of the gonads of either the male or females (Figures 4.20 and 4.21). Considering the testes of the male stickleback were at such rudimentary stages, it was quite difficult to identify any subtle abnormalities at this early stage. However, the mature three-spined sticklebacks sampled at 180 dph also did not appear to have any observable effects of DBP exposure on gonadal histology. In each of the three Experiments, the males from all treatment groups generally had reached the same developmental stage and had no visible abnormalities. By contrast, the females sampled at this time were frequently noted to have at least one atretic oocyte (Figure

4.23), but this phenomenon was also commonly observed in the control females and did not appear to be DBP-related.

Finally, the fish that were sampled for histology from Experiment 2 following the spawning study also showed no histological effects, except for a higher proportion of females with oocyte atresia (Figure 4.44). This was complicated by the fact that the most severe cases of this phenomenon were found in the Solvent Control group. The oocyte atresia encountered in the three-spined sticklebacks bore many similarities to the observations of the F_0 and F_1 fathead minnows exposed to DBP, since oocyte atresia was also noted in the Solvent Control groups (Chapter 2 Sections 2.33 and 2.34). As previously discussed, oocyte atresia has been an effect commonly noted in female rats and fish exposed to phthalates and other anti-androgens (see Chapter 2, Section 2.4.2.6). It is highly likely that such effects are related to the laboratory conditions rather than phthalate exposure, as suggested by Dr. Jeff Wolf (personal communication). Overall, histological analysis of the DBP-exposed three-spined sticklebacks suggested the gonads were able to produce mature gametes in normal proportions. Thus, this work did not confirm the findings of previously conducted experiments involving phthalate exposure in fish, including incidences of ovo-testis in males, or the significant reductions in the number of mature oocytes in females (Kim *et al.* 2002; Norman *et al.* 2006; Patyna and Cooper 2000; Patyna *et al.* 2006).

In the mature males from the Experiment 2 spawning protocol, there was no evidence of dysgenic testicular tissues similar to the abnormal seminiferous tubules commonly observed in male rats exposed to phthalates at doses >500mg/kg/day *in utero*. All testicular cysts appeared to have a normal structure, and there did not appear to be any apoptosis of germ cells that might suggest disrupted Sertoli-germ cell interaction. This was similar to the observations made in Chapter 2 (Sections 2.33, 2.34 and 2.35) in which the testes of F_0 and F_1 fathead minnow males also did not appear to have been affected by aqueous DBP exposure. It is possible that such observations were not noted in either the fathead minnows or the three-spined sticklebacks because the phthalate concentrations used in these studies were generally more than three orders of magnitude lower than the dietary doses required to induce such effects in male rats (Mahood *et al.* 2005; Mylchreest *et al.* 2002). On the other hand, it is important to recall that the route of exposure (dietary in rats and aqueous in fish) greatly affects the final internal concentration of phthalate. Thus, we cannot compare dietary and aqueous concentrations directly. It is possible that through bioaccumulation, the fish did managed to sequester comparable tissue concentrations of DBP as those in rats exposed to ~500 mg/kg/day. However, without knowledge of the internal exposure concentrations in the tissues of either animal, comparisons are extremely difficult.

Again, the lack of immunohistochemical techniques impeded the identification of any subtle effects DBP exposure may have had on Sertoli-germ cell interactions or Leydig cells in the testes, and oocyte atresia in the ovaries. In future, the development of such techniques would be useful to determine if DBP exposure has effects on the histology of the gonad in fish not observable with conventional staining techniques, and so commonly observed in rats exposed to DBP (Andrade *et al.* 2006; Barlow *et al.* 2004; Lin *et al.* 2008; Mahood *et al.* 2005; Mylchreest *et al.* 2002). However, I can say that DBP exposure early in life at concentrations ranging from 50 to >200 μg/L did not appear to interfere with the normal development of the gonad of the three-spined stickleback, and ultimately did not interfere with the production of viable gametes.

4.4.3 The effects of DBP exposure on plasma hormone concentrations

This was the first time that plasma oestrogen concentrations in females were measured in response to DBP-exposure in this project. In the female three-spined sticklebacks, the plasma oestradiol concentrations appeared to be unaffected by early life-stage DBP exposure in all of the experiments (Figures 4.30, 4.32, and 4.35). Only one other study conducted on fish has measured oestradiol concentrations in plasma sampled from females. Using questionably high aqueous concentrations of DEHP (5-20 mg/L), Han *et al.* (2009) found a concentration-related decrease in pooled plasma oestradiol samples (n=1) after exposing carp for only 48 hours in a recirculated exposure system. This finding was significant in the fish exposed to concentrations of 10, 15, and 20 mg DEHP/L.

Mammalian studies have reported equally confounding results in terms of oestrogen concentrations. Non-significant, dose-independent increases of ovarian oestradiol production were reported following exposure of rats to 500 and 1000 mg/kg/day DEHP from weaning to mating (Gray *et al.* 2006). Conversely, significant reductions in plasma oestradiol concentration have been measured in female rats exposed prepubertally, or as adults (Davis *et al.* 1994; Svechnikova *et al.* 2007). However, adult-stage inhalation of DEHP appeared to have no effects on plasma oestradiol (Ma *et al.* 2006).

In terms of androgens, DBP did seem to have some effect on the plasma androgen concentrations in male three-spined sticklebacks (Figures 4.22, 4.25, 4.26, 4.27, 4.29, 4.31, 4.33, and 4.34). However, it was difficult to determine a predominant effect, since both significant increases and decreases were observed with increasing DBP concentration. This was interesting, as increased plasma 11-KT concentrations had also been observed earlier, in both the F_0 and F_1 generations of fathead minnows exposed to DBP in Chapter 2 (Figures 2.16 and 2.28). As mentioned previously (Chapter 2, Section 2.4.2.7), similar findings have also been found in exposures of fish to phthalates *in vivo* (Han *et al*. 2009).

There are three reasons which might explain why such variable responses in plasma androgen concentrations were observed:

(i) There was a significant time lag between the period of exposure of the three-spined sticklebacks to DBP and the date on which the fish were sampled, which may mean that an immediate effect was missed.

(ii) DBP concentrations were possibly much too low to consistently reduce plasma androgen concentrations, since the effects of phthalates at lower concentrations are also more variable in mammalian studies.

(iii) Plasma hormone concentrations may not be the most consistent indicators of endocrine disruption in general.

In terms of the time-dependency of the effects of phthalate exposure, studies using rats have investigated the long-term effects on male offspring following *in utero* exposure, and found that testosterone concentrations tend, with time, to return to levels similar to those measured in control rats. For example, rats exposed prepubertally to >100 mg/kg/day DEHP *in utero* are reported to have significantly reduced plasma testosterone concentrations compared to controls immediately following exposure, but such differences are no longer apparent in adulthood (>90 days) (Akingbemi *et al*. 2001; Borch *et al*. 2004).

Overall, I would have liked to measure both androgen and oestrogen concentrations in the three-spined sticklebacks both during the DBP exposure period (0-24 dph) and also soon after its cessation. Unfortunately, the small size of the sticklebacks at these times made direct measurement of plasma hormone concentrations impossible. Instead, this would have required the measurement of concentrations from pooled whole-body homogenates, which would have used up several fish intended for other analyses.

Secondly, it is possible that the lack of consistency in the responses of plasma androgen concentrations in the male three-spined sticklebacks to DBP-exposure may be because the DBP concentrations were too low to consistently reduce plasma androgen concentrations. As reviewed in Chapter 2 (Section 2.4.2.7), the prevailing mammalian research often shows that at lower *in utero* phthalate concentrations plasma androgen concentrations are less predictable and often increase, while at higher concentrations they are more consistently reduced (Borch *et al.* 2006; Lin *et al.* 2008). Thus, the trends of both increasing and decreasing plasma androgen concentrations in the three-spined sticklebacks exposed to DBP may represent similar changes in plasma androgens to the responses to lower dietary exposure concentrations in mammalian studies. However, as stated previously, we cannot directly compare dietary mammal exposures to aqueous exposures in fish without any knowledge of the tissue concentrations. Thus, this is simply conjecture; we can only suggest that it is possible a higher DBP concentration might lead to more consistently reduced plasma androgen concentrations in male three-spined sticklebacks.

Finally, it is also possible that the analysis of plasma androgen concentrations may not be the most appropriate endpoint for the analysis of endocrine disruption. This may be due to the fact that plasma hormones are either too variable, too sensitive to other factors, and/or because the mechanism of action of phthalates is unknown. (For example, an anti-androgen that antagonized the androgen receptor may not necessarily be expected to result in decreased plasma androgen concentrations). In contrast to plasma testosterone concentrations, *ex vivo* testosterone production in rats is generally reduced following *in utero* phthalate exposure, but not exclusively so (Akingbemi *et al*. 2004; Akingbemi *et al*. 2001).

Similarly, studies with fish using different anti-androgenic chemicals have also been unable to pin down consistent responses of plasma hormone concentrations. For example, exposure of the fathead minnow to the anti-androgenic fungicide, ketoconazole caused significant reductions in fecundity, spawning frequency, and significant increase in GSI, but was unable to alter plasma oestradiol concentrations in females or plasma testosterone concentrations in males in any notable manner. This is especially surprising since ketoconazole is thought to directly interfere with testosterone production (Ankley *et al*. 2007). In another study, exposure of adult fathead minnows to 50 or 500 μg /L of the potent anti-androgen, flutamide (an androgen-receptor antagonist), for 21 days had no effect on plasma testosterone, 11-KT, or oestradiol concentrations in male fish. In this case, the female fish also had no changes in their plasma 11-KT and oestradiol concentrations, but did have significantly higher plasma testosterone concentrations compared to controls at the 500 μg/L concentration (Jensen *et al*. 2004). Another study, exposing adult fathead minnows to vinclozolin (a chemical whose metabolites also antagonise the androgen receptor) for 21 days (200 and 700 μg/L), also found no effect on plasma hormone concentrations (Makynen *et al*. 2000). Thus, exposure to more potent anti-androgens than phthalates at higher concentrations than those used in the current work, were also unable to elicit a consistent response in plasma hormone concentrations in fish species. Perhaps even more interestingly, another study found that exposure of adult fathead minnows to vinclozolin (at concentrations of 60, 255, and 450 μg/L) for 21 days, did not cause any changes to plasma testosterone concentration in males, but did significantly disrupt *ex vivo* testicular testosterone production at all exposure concentrations compared to controls (Martinovic *et al.* 2008).

It also would have been very useful to measure the *ex vivo* production of steroid hormones by the excised gonads in the current work, since this appears to be a more consistent response to DBP in mammals and to other anti-androgens in fish (Akingbemi *et al.* 2004; Martinovic *et al.* 2008). Unfortunately, due to time-constraints this method was not adapted for the three-spined stickleback. Consequently, the effects of phthalate-exposure on *ex vivo* androgen production in the three-spined stickleback remains unknown.

In general, the results suggest that DBP exposure in early life does affect the circulating concentrations of androgens in male three-spined sticklebacks, but exactly in what way remains uncertain. Ultimately, these hormonal differences did not affect the ability of the fish to spawn successfully in the laboratory.

4.4.4 Nuptial colouration

Nuptial colouration in males has commonly been studied by behavioural zoologists and ecologists for its relation to body condition and female mate choice (Bakker 1994; Frischnecht 1993; Milinski and Bakker 1990). The three-spined sticklebacks are unable to produce carotenoid pigments themselves, but rather sequester them from food (Barber *et al.* 2000). While the intensity and area of nuptial colouration is androgen-mediated (Mayer *et al.* 1990), it can also be affected by several factors including parasite-load, body condition, predation, and competition with other males (Barber *et al.* 2000; Candolin 2000). A previous endocrine disruption study found early life-stage exposure to an oestrogen induced a non-significant decrease in the proportion of red in male throats as calculated by r=R/(R+G+B) (Maunder 2006). Similarly, exposure of the guppy (*Poecilia reticulata*) to the anti-androgens vinclozolin, *p,p'*-DDE, and flutamide from hatch to maturity also caused reduced redness in the male orange colouration (Bayley *et al.* 2002). By contrast, Candolin *et al.* (2000) found that while the intensity of the red colouration was unaffected by the introduction of male competition, the area of redness did change, and that this area was a more "honest" index of the condition of each male when male-male competition was present. The findings of Barber *et al.* (2000) made similar observations by comparing the intensity and extent (relative area) of the red colouration of 58 male three-spined sticklebacks when they were caught in the wild, to their expression following a two-week period in the laboratory. Initially, both the extent and intensity of the colouration in the field-caught males were found to correlate strongly with body condition. The extent of the redness also correlated negatively with white blood cell counts, although this correlation was considered only marginally significant. However, following the period in the laboratory, in which the males were kept in individual tanks and visually isolated from each other, the extent and intensity of their red colouration no longer correlated to body condition or white blood cell counts. Further, they found the variation of extent and intensity between males had decreased, and that the dullest males analysed in the field had increased the most in the intensity and extent of their colouration after two weeks in the laboratory. The authors concluded that the analysis of nuptial colouration in the three-spined sticklebacks is only an accurate indicator of body condition when measured in the field (Barber *et al.* 2000).

In the current study, the redness of throat failed to show any relationship with DBP exposure (Figures 4.36 and 4.38). Further, when the throat colouration of the Experiment 2 and 3 males was correlated with either plasma androgen or spiggin concentrations (Experiment 3 only), the redness of the throat did not correlate particularly strongly with either spiggin, 11-KT or testosterone concentrations (eg. Figure 4.37). Thus, we could not provide any strong support for the hypothesis that the redness of male throats is directly related to circulating androgen or spiggin concentrations. This may be due to the lack of standardized conditions used to photograph some of the males. However, our negative results suggest several possibilities. First, red nuptial colouration of the throat may not be sensitive enough to the weak anti-androgenic activity of phthalates or the diet of the males in these experiments may not have contained enough carotenoids for them to produce a more intense signal. Second, our methods for the analysis of nuptial colouration may have been inadequate to detect any subtle difference in colouration, for example, the use of improper light sources while photographing the fish. It may be more useful, in future, to extract the pigment from the jaw area to analyse the carotenoid pigment astaxanthin as described in Barber *et al.* (2000). Finally, it is possible that the lack of any effects of DBP on nuptial colouration was because the conditions under which the males were kept were inappropriate for the development of "honest" nuptial colouration in relation to DBP (Barber *et al.* 2000; Candolin 2000).

Overall, the results of this work lead us to the conclusion that exposure to phthalates in early life did not change the redness of throats in sexually mature males, but that the analysis of nuptial colouration in three-spined sticklebacks may be too sensitive to confounding factors to provide any significant insight into the effects of phthalate-related endocrine disruption.

4.4.5 Spiggin and kidney epithelium height

In Experiment 1 males, the difference in concentrations of spiggin between treatment groups was not significant, but may have been due to a low sample size in the Solvent Control since there was a strong spiggin concentration-related increase with DBP-treatment when pooled (P=0.064)(Figure 4.39). In this instance, spiggin concentrations also correlated strongly with plasma 11-KT concentration (Figure 4.41a). By contrast, spiggin concentrations in Experiment 3 males appeared to be unrelated to DBP treatment and were not correlated particularly closely with the plasma 11-KT concentrations ((Figure 4.41b). In Experiment 2, the males exposed to DBP also had no discernible differences in kidney epithelium cell height compared to the Solvent Control fish, and cell height was not closely correlated to plasma 11-KT concentrations (Figure 4.42).

It is highly possible that the concentrations of spiggin in Experiment 1 males were a direct response to exposure to DBP in early life. However, the reason as to why spiggin concentrations were increased as opposed to decreased, and why this response was not replicated in the males in Experiments 2 and 3, is unknown. It may be possible to attribute the lack of a significant finding in the spiggin concentrations of Experiment 1 males to the small sample size of the Solvent Control. If this argument is correct, we would assume that the males exposed to DBP in early life were able to more rapidly respond to the increasing day length and light intensity, and to produce spiggin much earlier than males that were not exposed to DBP. Based on mammalian studies, it is perhaps the increased number of Leydig cells induced by DBP that may facilitate this rapid response. However, with no evidence of such an occurrence in the three-spined stickleback, it is difficult to say whether or not this response is repeatable, and if so, what the underlying cause of such a response is. Perhaps instead of negating this finding from Experiment 1, the results from Experiments 2 and 3 may suggest that, eventually, all males were able to produce spiggin at similar concentrations regardless of DBP-exposure. Until this experiment is replicated, however, the effect of early life-stage exposure of DBP on spiggin concentrations remains unclear.

4.4.6 Nest building and spawning success

The results from the nesting study of Experiment 3 fish provides the only reliable data on the effects of early life-stage exposure to DBP on the timing of nest-building in males induced to spawn. It is impossible to draw major conclusions based on the results, especially due to the small number of males in the Solvent Control group (n=3). These males took several days longer than fish in the 100 and 200 μg DBP/L groups to build a nest, but this delay was not statistically significant (Figure 4.28). When these data are considered alongside our findings of increased concentrations of both spiggin and plasma androgen in DBP-exposed males in the first nesting study from Experiment 1 (Figures 4.29 and 4.39), this supports the hypothesis that males exposed to DBP in early life are able to reach the breeding condition more quickly than control males. However, the nest-building activity starkly contrasts with the onset of spawning activity in the F_0 fathead minnows (Chapter 2, Figure 2.21) in which spawning occurred the earliest in the Negative and Solvent Control groups and latest in the 50 and 100 μg DBP/L groups. Overall, this requires more investigation.

In general, the results of the Experiment 3 nesting study showed that, in the absence of competition, male three-spined sticklebacks exposed to DBP in early-life were as likely to build nests as control males. This conclusion was further supported with the 100% nest building success in Experiment 2 males within all treatments groups.

The spawning study conducted in Experiment 2 was complex and the results remain difficult to interpret in detail. Under such unnatural conditions, the mating success of each male was mainly dependent on the willingness of each female. When we consider that the number of ovulating females was highly variable between treatment groups, and that the study period could have been further extended to give more males the opportunity to spawn, it is impossible to draw conclusions on the effect of DBP when comparing how many males spawned in one group versus another. What we can conclude, however, is that males from each group were able to spawn, and that viable offspring were produced with similar frequency across treatments. Therefore, DBP exposure during early life did not impair the ability of the three-spined stickleback to successfully reproduce in the laboratory.

In future, it would be very useful to test the ability of male three-spined sticklebacks exposed to DBP in early life to successfully compete with control males for female mates. It would also be interesting to assess the nest quality in this experiment, since nest neatness and compactness has been found to correlate positively with relative kidney weight and negatively with relative spleen weight (an indicator of immune stress (Barber et al. 2001). Further, a pair-breeding study with another fish species, such as the fathead minnow, would also likely be quite useful in determining if fish exposed to phthalates during early-life stages have altered fecundity compared to control males. This would be especially interesting since a previous pair-breeding study exposing fathead minnows to a different phthalate, BBP (69 and 82 μg/L for 3 weeks), found that the frequency of spawning events significantly decreased. However, the number of eggs per spawn also increased, and thus fecundity *in toto* was not affected by BBP-exposure (Harries *et al*. 2000).

4.5 Conclusions

This series of experiments demonstrated that three-spined sticklebacks exposed to environmentally-relevant concentrations of DBP during a critical period in development are able to alter plasma androgen and spiggin concentrations, but that such responses are inconsistent and poorly understood.

Overall, DBP did not appear to impair the reproductive development of sticklebacks at concentrations commonly measured in the environment. The three-spined sticklebacks exposed to DBP in early life appeared to develop normally, and were able to reproduce at a similar rate of success as the males in the Solvent Control groups. However, DBP did appear to elicit significant effects on plasma androgen concentrations in males at 130 and 180 dph, and also after developing into the breeding condition. Further, spiggin concentrations were also markedly increased in some of the groups of males that were exposed to DBP and used in the nesting studies. As a whole, further research is required to elucidate the nature of the endocrine disrupting activity of DBP in fish.

Chapter 5. Adult-stage exposure of the three-spined stickleback male to DBP

5.1 Introduction

In order to properly investigate the potential effects of phthalates on fish, it was necessary not only to determine if DBP affected fish that were exposed during critical periods in development, but also if it was able to cause endocrine disruption in adult fish.

In order to investigate this, an experiment was developed to expose mature males to environmentally-relevant concentrations of DBP in a nest-building protocol. The experimental system was based on the design used in Chapter 4, originally developed by Dr. Ioanna (Katsiadaki *et al.* 2007). In brief, quiescent male three-spined sticklebacks were placed into individual tanks for 3 weeks and exposed to various concentrations of DBP, while simultaneously being brought into the breeding state by increasing light intensity and photoperiod. The aim was to investigate whether or not DBP exposure would affect the timing of development of the breeding condition, concentrations of sex hormones in the plasma, concentrations of the androgen-dependent spiggin protein, the expression of male nuptial colouration, and nest-building and courtship behaviours. In addition to these parameters, we also aimed to investigate the effects of DBP on the expression of steroidogenic genes in the testes. This study presented the most likely case in which phthalate exposure would lead to measurable changes in the expression of steroidogenic genes in fish, since the up- or downregulation of genes caused by chemical exposure is observed during or soon after exposure (Struve *et al.* 2009). Therefore if a change in steroidogenic gene expression were to be caused by exposure to DBP, it seemed more likely that it would be observed in a short-term study such as this, than in the longer-term exposures reported in Chapters 2 and 4.

The downregulation of the level of expression of several steroidogenic genes following phthalate-exposure has been a commonly reported effect in male rats exposed *in utero* (Barlow *et al.* 2003; Borch *et al.* 2004; Liu *et al.* 2005; Plummer *et al.* 2007; Shultz *et al.* 2001; Wong and Gill 2002). Among the genes controlling steroidogenesis in vertebrates are 3β-hydroxysteroid dehydrogenase (3β-HSD), and steroid acute regulatory protein (StAR). StAR is a protein that regulates steroid synthesis by controlling the transport of the steroid precursor, cholesterol, into the mitochondria. Cholesterol is then rapidly converted to pregnenolone by P450 side-chain cleavage enzyme (P450scc) and follows an equally rapid conversion to sex hormones (Stocco and Clark 1996) (Figure 5.1). To demonstrate the importance of StAR, infants with Congenital Lipoid Adrenal Hyperplasia lack a functioning StAR. Consequently they suffer from several symptoms, including feminized external genitalia due to a lack of testicular testosterone (Lin *et al.* 1995).

The hydroxysteroid dehydrogenases, including 3β-HSD, are catalytic enzymes involved in the reaction converting hydroxysteroids to ketosteroids. 3β-HSD, specifically, is essential in the production of not only sex steroid hormones, but glucocorticoid and mineralcorticoid hormones as well. It converts pregnenolone to progesterone, but is also involved in the enzymatic conversion of other steroid hormones downstream. Human deficiency in 3β-HSD is the cause of severe hypospadias and undifferentiated gonads in males, and hyperandrogenism in females due to the accumulation of the androgen, dihydroepiandrosterone (Codner *et al.* 2004; Heinrich *et al.* 1993).

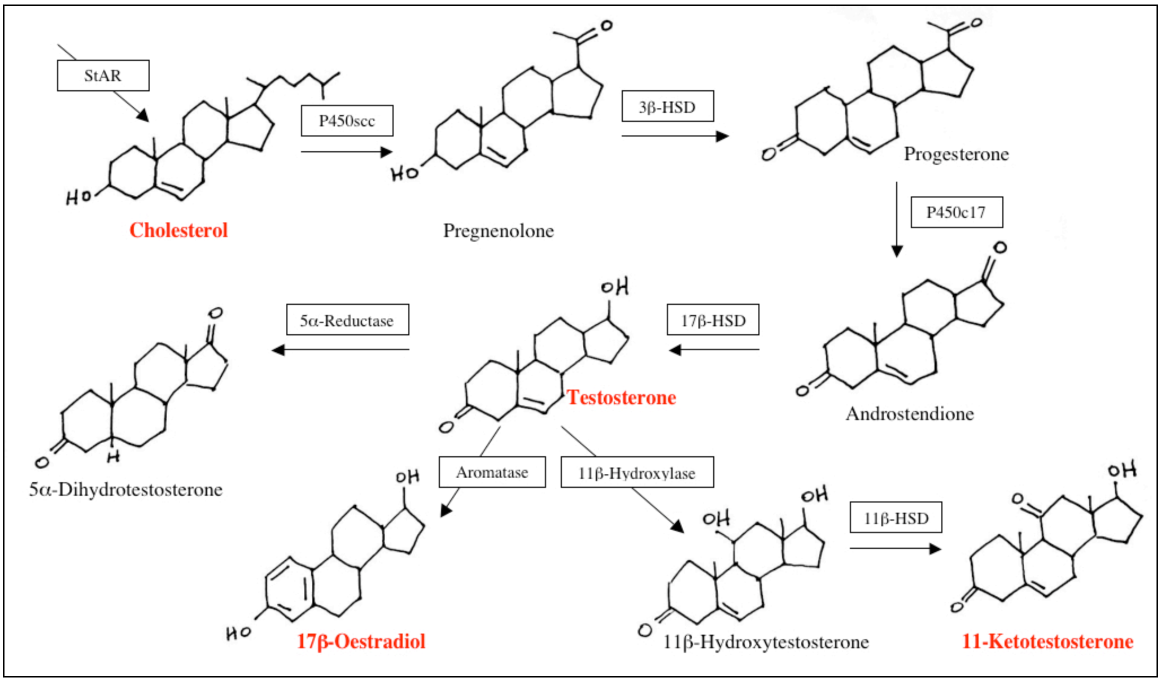


Figure 5.1. A simplified depiction of steroid biosynthesis in the teleost fish. The steroid hormones of particular interest are marked in red, and the enzymes and proteins involved in the process are enclosed in boxes. Adapted from (Branch 2001; Kime 1998).

As discussed in Chapter 1, high-dose phthalate exposure in mammals has been linked to reduced testosterone concentrations in the testes and in blood plasma, and also to reduced expression of several steroidogenic genes. In these studies, StAR is more commonly analysed than 3β-HSD, but the latter may be more sensitive to disruption. One study found significant downregulation of 3β-HSD in fetal rats (on Gestation Day 19) exposed to doses of ≥0.1 mg DBP/kg/day *in utero*, while StAR was only significantly affected at ≥50 mg/kg/day (Lehmann *et al*. 2004). However, not all studies are in agreement: some investigations of *in vivo* phthalate effects have also found significant upregulation of StAR (Culty *et al*. 2008; Lahousse *et al*. 2006; Lee *et al*. 2009; Ryu *et al*. 2007).

To date, the effects of phthalate exposure on the expression of steroidogenic genes in fish has not been investigated. In this study, 3β-HSD and StAR were chosen instead of several other steroidogenic genes for specific reasons. 3β-HSD was chosen because it appears to be fairly sensitive to phthalate disruption in rats (Lehmann *et al.* 2004). StAR was chosen because, firstly, DBP, as a peroxisome proliferator, may have a direct role in cholesterol transport. This effect could be related to the expression of StAR , since its role in steroidogenesis is also cholesterol transport (Gazouli *et al.* 2002). Secondly, StAR is commonly downregulated in male rat offspring following *in utero* phthalate exposure. The expression of 3β-HSD and StAR were measured in the testes of exposed adult three-spined sticklebacks using real-time RT-PCR.

5.1.1 *Experimental aim*

The aim of this experiment was to test whether or not DBP exposure would reduce the production of androgens in the male fish, causing delays in the onset of nest-building, reduced plasma androgen and spiggin concentrations, diminished redness of the nuptial colouration of the throat, and downregulate the expression of the genes 3β-HSD and StAR, involved in steroidogenesis.

5.2 Methods

5.2.1 *Animal husbandry*

Three-spined sticklebacks (n≈80) were obtained from the wild on August 19th, 2008, from a series of ponds located in Hook, Hampshire, UK (51°18' N 0°55'W). A small stream occasionally feeds these ponds (Moore & Moore Carp Suppliers, personal communication).

Upon arrival at the laboratory facilities at Brunel University, fish slowly acclimatized to the new conditions. Based on the visual characteristics that developed over 4 weeks, they were separated into Female, Male, and Mixed Stock fish tanks. The fish density was kept at approximately one fish per litre to discourage dominance hierarchies in the tanks.

The three-spined sticklebacks were initially maintained in "summer" conditions at 18°C, with a photoperiod of 16h light and 8h dark and light intensity $\geq$ 600 lux in all tanks. Fish were fed twice daily with frozen bloodworm (*Chironomidae*). The fish in the Male tank were subjected to "winter" conditions from the end of November onwards by reducing the photoperiod and light intensity to 8h light: 16h dark and 50 lux, respectively, and temperatures of approximately 18-20°C.

5.2.2 *Glugea anomala infection*

For the first few months, all wild-caught fish appeared healthy and were feeding normally. However, on November 5th, 2008, one dead fish was discovered in the Male tank. Nine fish died in the Mixed Stock tank in the following two weeks, and the cause of the mortalities was soon identified as a common stickleback microsporidian parasite, *Glugea anomala*. This was diagnosed after finding a fish in the Mixed Stock tank with a large white growth on its caudo-lateral area (Figure 5.2) (Noga 1996). Both the fish and the growth were preserved for histology (for methods see Chapter 2, Section 2.2.9). A fish pathologist analysed the histological samples to confirm the diagnosis (Figure 5.3) (Dr. Chris Williams, Environment Agency, UK, personal communication).

In November 2008, these three tanks of wild-caught three-spined sticklebacks were quarantined to ensure the lab-reared three-spined sticklebacks (used in the experiments described in Chapter 4) were not infected. In other words, no physical contact (via tools, food, water) occurred between the laboratory-reared and wild-caught three-spined sticklebacks. All of the tanks of wild-caught three-spined sticklebacks were exposed to 3 courses of the anti-parasitic drug, mebendazole (Sigma Aldrich Ltd.); this was dissolved in acetone (Fisher Scientific Ltd.) and added to the static tanks at a nominal concentration of 2 mg mebendazole/L water, for 6 hours every 2 days for 6 days. One fish died following the first exposure in the Mixed Stock tank, with no subsequent mortalities.

Figure 5.2. Photograph of a three-spined stickleback with a subcutaneous growth (arrow), later diagnosed as *Glugea anomala*, a common microsporidian parasite of *Gasterosteus spp.*

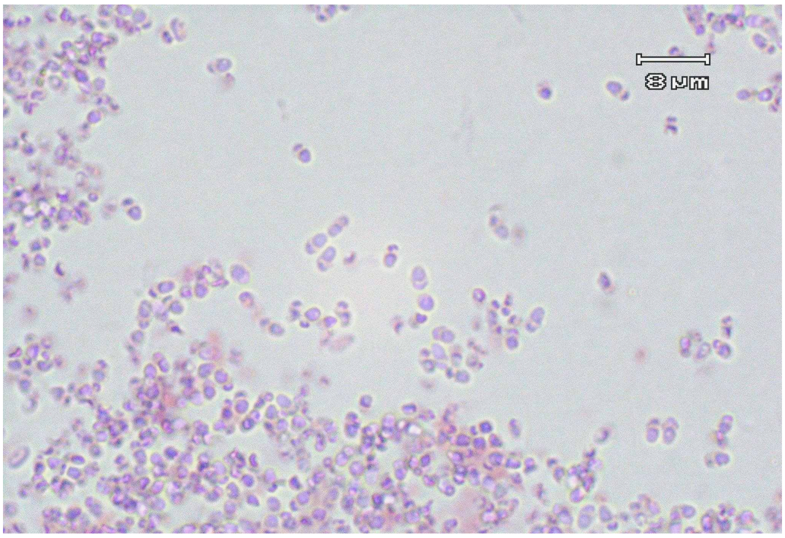


Figure 5.3. Slide of the microspores of *Glugea anomala* from the growth removed from the three-spined stickleback shown in Figure 5.2 (taken by Chris Williams, Environment Agency, UK).

Mebendazole was analyzed for endocrine activity with the Yeast Androgen Screen by Dr. Alice Baynes at the Institute for the Environment. Mebendazole was found to have 1/1000 the potency of dihydrotestosterone in its ability to activate androgen receptors. Mebendazole is a fairly soluble drug with a log K_{ow} of 2.14. In humans, it has been documented to be metabolised within a few hours of exposure into much more hydrophilic metabolites (Braithwaite *et al*. 1982). It is likely that mebendazole and its by-products were metabolised by the three-spined sticklebacks well-within the 104 days between receiving treatment and the beginning of the experiment, which was not started until March, 2009.

To ensure the *Glugea anomala* had been eradicated from the stickleback population, 13 three-spined sticklebacks were randomly sampled from the Mixed Stock tank for pathological analysis after the experimental study had been completed. *Glugea* generally requires 3-4 months to develop in three-spined stickleback fish. Therefore, if the parasite was present, it was given enough time to reach a stage at which it could be properly diagnosed. Each fish was anaesthetized in MS-222 and pithed, then a slide coverslip was used to scrape the lateral side of the body. The fin-scrape was prepared as a wet-mount and examined under a compound light microscope, along with squash preparations of spleen, liver, and kidney. The gut contents and eye were examined in saline solution under a dissecting microscope for any other parasites, such as nematodes. In all 13 fish, there did not appear to be a *Glugea anomala* infection and no other parasites were identified. Further, no other fish were lost after the tanks had been treated with mebendazole.

5.2.3 *Exposure of adult three-spined sticklebacks to DBP in a nesting protocol*

In this experiment 24 adult three-spined stickleback males were exposed to three treatments: a Solvent Control, and nominal concentrations of 50 and 100 μg DBP/L for 22 days (March 9th to 31st, 2009, n=8 per treatment). The experimental system was set up 3 days prior to the beginning of the experiment, to allow the concentrations of DBP to stabilize before adding the fish. The flow-through tank system, peristaltic pump, and flow rates were set up in the exact same manner as in Chapter 4 (Section 4.2.1). The only difference was that, instead of Mixing Vessels, three distribution trays were used (one per treatment group). These distribution trays mixed the incoming water with the incoming stock solutions, as before, but then distributed the water by gravity into nine separate tanks: eight fish tanks (3.8 L each) and one Analytical Chemistry tank (8 L, with no fish) (Figure 5.4). Each fish tank had an approximate flow rate of 3.33 L/h.

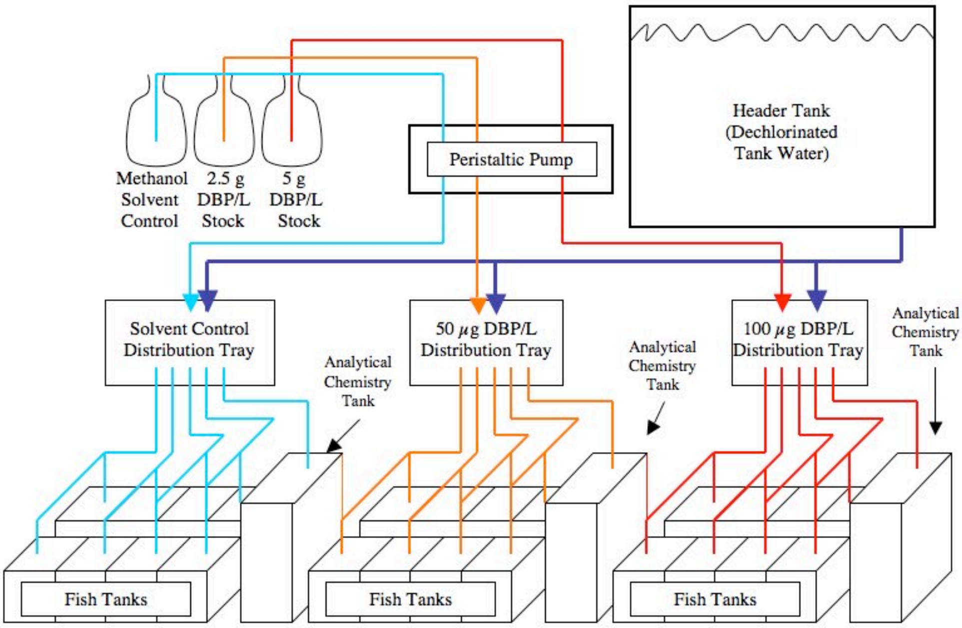


Figure 5.4. Representation of the experimental system used to expose 24 adult male sticklebacks to various concentrations of DBP in a 22-day nesting study (n=8 per treatment).

5.2.4 *Water chemistry*

Due to the small size of the exposure tanks, and to minimize disturbance of the fish, the "Analytical Chemistry Tanks" were used for water sampling (Figure 5.4). All water samples were collected in duplicate every 3-5 days. Volumes of 500 ml were collected from the Solvent Control and 50 μg DBP/L Analytical Chemistry tanks, and volumes of 250 ml were collected from the 100 μg DBP/L tank. All samples were kept at 4°C until they were liquid-liquid extracted. This procedure was conducted within 3 hours of sampling, according to the optimized method described in Chapter 4, Section 4.2.1.1, and run on the GCMS as previously described (Chapter 2, Section 2.2.6.2).

5.2.5 *Nesting protocol*

The nesting protocol was conducted as described in Chapter 4 (Section 4.2.7). Twenty-four males were selected from the Male stock tank and added to a large container filled with fresh tank water. Each male was then added to one of the 24 experimental tanks using a table of random numbers, indicating the tank into which each fish was added. Special care was taken to ensure that the males selected for this experiment excluded any coloured (dominant) males, which might bias the results.

After the fish were added (Day 0), the photoperiod was extended to 16h light:8h dark per day, and the light intensity was increased to approximately 1000 lux. The temperature was maintained at 20°C, and each tank was aerated. Fish were fed three times daily, twice with frozen bloodworm and once with ZX 400 Fish Food (ZM Systems Ltd.). The males were left to acclimatize to these new conditions for the first 10 days of the experiment. On Day 10, nest-building material was added and males were visually isolated from one another. Nest building was documented in each tank and recorded with a photograph every second day. On days 16 and 20, the males were visually exposed to an adult female contained in a beaker for 5 minutes. On Day 22, the experiment was terminated.

Sampling of the fish on Day 22 involved anaesthetizing each male. The ventral and right sides of the head were then photographed. Blood was sampled from the caudal peduncle, and fork length and wet weight were measured. The fin tissue and gonads were removed, weighed, and snap frozen in liquid nitrogen for subsequent analysis of gene expression. The kidney was also excised for the measurement of spiggin concentration and stored first, on ice and at -20°C thereafter. The blood samples were again, spun in the centrifuge at 12,500 g for 5 minutes, and blood plasma drawn off and decanted into clean vials. The ethyl acetate was added to the plasma aliquots at a ratio of plasma to ethyl acetate of 1:220. All samples were stored, processed, and analysed in the exact same manner as described previously (see Chapters 2 and 4).

5.2.6 *Real-time PCR protocol*

5.2.6.1 Introduction to real-time PCR

Real-time RT-PCR (qRT-PCR) was employed, since it is the most accurate method for the quantification of gene expression. In brief, qRT-PCR is a sensitive assay, which quantifies the amount of messenger RNA in a sample. Messenger RNA (mRNA) is the template upon which proteins, including enzymes, are synthesized. Generally, an mRNA strand is specific for a certain protein, and each one is transcribed directly from a specific DNA-sequence, or gene. The more mRNA present, the more copies of the protein are built and thus, the more "expressed" the gene. Thus, there is a direct relationship between the amount of mRNA present and the degree of expression of a gene.

Since mRNA is very fragile, for measurement in the laboratory it is first converted to complimentary DNA (cDNA), so that it can be used in qRT-PCR without degrading. This conversion ratio is 1 to 1; the amount of cDNA quantified is equivalent to the original amount of mRNA. The process of real-time RT-PCR is based on the same concept as normal RT-PCR. Primers target the specific cDNA fragment that derives from the gene of interest. The fragments are copied using Taq polymerase which creates two double-stranded copies for every double-stranded fragment. Thus, for every amplification cycle, the number of target fragments increases exponentially. Real-time RT-PCR is special, since it is able to measure the original amount of cDNA in the sample by amplifying it along with a fluorescent marker, SYBR Green. The SYBR Green binds to double-stranded DNA fragments, and when it does it fluoresces. Hence, the amount of fluorescence in a sample is relative to the amount of the cDNA fragments produced in each amplification cycle. By monitoring the number of cycles it takes to produce a certain amount of fluorescence, we can back-calculate the original amount of mRNA (via cDNA) present. The more mRNA an exposed fish has compared to the control fish, the more expressed the gene, which is thus considered to be “up-regulated”.

The real-time PCR analysis method involved several steps:

1. Extraction of the RNA from the tissue of interest, in this case the testes.
2. Conversion of the extracted mRNA fraction of the sample to cDNA.
3. Verification of the quality of the cDNA in a normal RT-PCR using a well-established primer pair (in our case, the housekeeping gene, β-actin).
4. Design of primers for both the target genes as well as housekeeping genes. This also involves checking the primers by RT-PCR with the cDNA template, and verifying the product sequences are correct by DNA sequencing to ensure the primers are targeting the correct genes.
5. The optimization of RT-PCR by preliminary runs verify that the most favourable conditions and component concentrations are employed.
6. Producing efficiency curves for each primer set to determine the specific efficiency of each primer pair to be used in the analysis of results.
7. Running the real-time PCR of the cDNA samples, run with the target primers (3β-HSD and StAR), as well as with the housekeeping genes (Ubiquitin and β-actin).

5.2.6.2 RNA extraction

Ribonucleic Acid (RNA) was isolated from each of the 24 excised testes of the male three-spined sticklebacks exposed to various concentrations of DBP for 22 days using the Rneasy Kit (Qiagen Ltd.). The gonads were homogenized in lysis buffer and β-mercaptoethanol using an electric Rotor Homogenizer (Fisher Scientific Ltd.) for 30-40 seconds. The homogenizer was washed in between samples by running it in sterile deionized water, 100% ethanol, and sterile deionized water once more. These liquids were replaced after every three samples to ensure no carry-over occurred. The tissue homogenate was mixed with 70% ethanol to precipitate out the RNA, which was collected on a column, washed, and eluted according to the kit instructions. Once completed, the purity of each RNA sample was measured by spectrophotometry (Nanodrop, Fisher Scientific Ltd.). All samples were stored at -80°C.

5.2.6.3 Conversion of mRNA to cDNA and the verification of cDNA

Once all 24 samples were extracted, the mRNA of each sample was converted to cDNA using the Superscript II First-Strand Synthesis Kit (Invitrogen Ltd.) on the iCycler PCR Instrument (Bio-Rad Laboratories Inc.). This involved taking 5 μg of mRNA from each sample and running it in a 50 μl PCR tube with several components to convert it to cDNA. Once finished, the cDNA samples were stored at -20°C. However, to verify the presence of an adequate amount of cDNA in each sample, all cDNA samples were run by RT-PCR with β-actin primers. These were designed by Dr. Tamsin Runnalls (Institute for the Environment) for use in the fathead minnow with the sequences Forward 5' – GAT ATG GAG AAG ATC TGG C- 3' and Reverse 5' – GTT GGC TTT GGG GTT CAG G – 3'. These sequences were checked for homology with the same gene in the three-spined stickleback using NCBI Basic Local Alignment Search Tool (BLAST) and were both found to be a 100% match with a partial sequence of β-actin in *Gasterosteus aculeatus* (DQ018719.1). The size of the PCR product in the three-spined stickleback was expected to be 101 bp.

The Accuprime Taq DNA Polymerase System (Invitrogen Inc.) was used for replication as in Chapter 4 (Section 4.2.8.2). Each sample was prepared in a sterile thin-walled PCR tube on ice according to the following method:

1 μl cDNA samples + 1 blank
\+
Prepared as Mastermix and added 24 μl to each sample:
2.5 μl 10X Buffer I
0.25 μl Forward Primer (10mM)
0.25 μl Reverse Primer (10mM)
0.5 μl Taq polymerase
20.5 μl dH$_2$0 (autoclaved)

DNA amplification was conducted on the iCycler using the following thermal program:

94°C for 5 minutes
94°C for 30 seconds
58°C for 30 seconds
72°C for 30 seconds
Repeated for 30 cycles
72°C for 5 minutes
4°C for ∞

Following replication, a 1% agarose gel was prepared as described in Chapter 4 (Section 4.2.8.2). The samples were then mixed with 2 μl loading buffer, and 12 μl of each sample was pipetted into the appropriate well alongside a 100 bp ladder (100-1500 bp, Invitrogen Inc.) and run in an electrophoresis chamber at 80V. After 45 minutes, the gel was analyzed with a UV light to check for bands 101 bp in length, and with similar fluorescence (Figure 5.5). A band of this size indicated replication of the β-actin sequence from the cDNA samples, and the similar fluorescence indicated little variation in the amount and quality of cDNA in each sample.

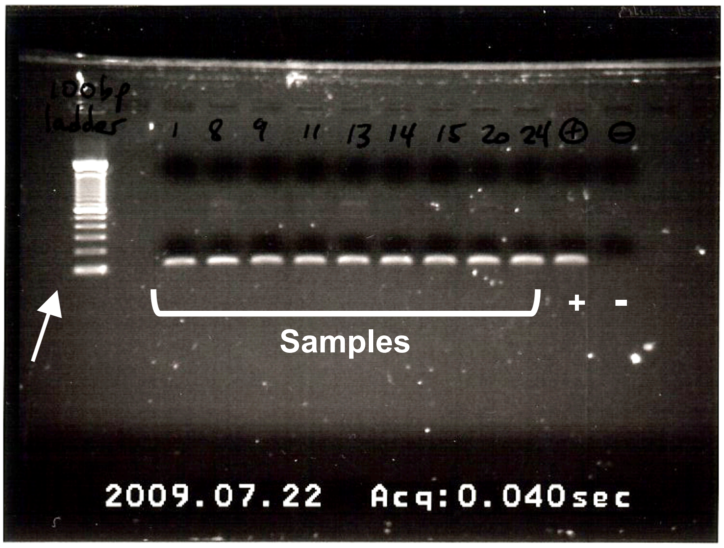


Figure 5.5. Photograph of an agarose gel under UV-light, loaded with amplified products of cDNA samples from *G. aculeatus,* run with primers for the β-actin gene. These bands all have a relatively similar fluorescence and product size ~100 bp, as indicated by the ladder (arrow). The samples are indicated by the brackets, and the + and – symbols indicate the positive and negative controls).

5.2.6.4 Real-time RT-PCR primer design and verification

Primers were designed for both 3β-HSD and StAR. The Primer Quest program (http://eu.idtdna.com / scitools / applications / primerquest / default.aspx) was used to design primers for the gene sequences of 3β-HSD and StAR in *Gasterosteus aculeatus* from the online database at http://www.ensembl.org (ENSGACT00000001847 and ENSGACT00000015623, respectively). Parameters were set according to the guidelines outlined by Premier Biosoft (http://www.premierbiosoft.com / tech_notes / PCR_Primer_Design.html). In brief, each primer pair was designed to be 18-22 bp in length, and to have melting points

(T_m) between 52-58°C and less than 1°C from one another, a GC content of 40-60%, a low Gibbs Free Energy for the formation of heterodimers and primer-dimers (ΔG of > -5 and > -2 kcal/mol, respectively), and amplicon lengths of 90-150 bp. The 3β-HSD primers were redesigned when initially no product could be replicated by RT-PCR. The resultant primer sequences for 3β-HSD and StAR are shown in Table 5.1.

Table 5.1. The forward and reverse primer sequences used to measure the expression of 3β-HSD and StAR by qRT-PCR in *Gasterosteus aculeatus*. Melting points (T_m) and the size of their product are indicated to the right.

Gene	Primer Sequence (5' → 3')	T_m (°C)	Product Size
3β-HSD	Fwd – TCA GTT ACT CGG ACT TCA ACC ACG	58.3	112 bp
	Rev – ACC TCC ATC AGG AAG CAG AAT GAG	58.3	
StAR	Fwd – GGT GGG ACC GAG GGA CTT TG	59.8	91 bp
	Rev – GTT GGT GTG CTG AGT TGA CAT T	59.9	

Primers for Ubiquitin were also used, as this gene was recommended as a useful housekeeping gene in the three-spined stickleback (Hibbeler *et al*. 2008). Ubiquitin primer sequences, designed by (Hibbeler *et al*. 2008), were Fwd 5' – CAG GAC AAG GAA GGC ATC C – 3' and Rev 5' – AGA CGG GCA TAG CAC TTG C – 3' with melting temperatures of 58 and 57°C, respectively, and an amplicon length of 215 bp.

All primer sets were run by RT-PCR with cDNA, according to the methods in Section 5.2.6.3. The bands identified by gel electrophoresis were verified to be of the correct length in all cases (data not shown).

5.2.6.5 Gene sequencing

The specificity of the StAR, 3β-HSD, and Ubiquitin primers were verified by sequencing the fragments they amplified using DNA Sequencing & Services, Dundee University, UK. In brief, amplified cDNA products were isolated by gel electrophoresis and purified using the MinElute PCR Purification Kit (Qiagen Ltd.). They were then prepared according to the Sequencing Service instructions and sent via mail to be analysed.

For each primer pair, the sequences of both forward and reverse primers were compared directly to their target gene sequences using the Ensembl.org BLAST (at http://www.ensembl.org). Both Ubiquitin primers yielded products of 189 and 194 bp, close to their target of 215 bp. The BLAST analysis reported that both products were 86-96% similar to the Ubiquitin gene in other organisms, and 99% similar with the Ubiquitin mRNA sequence in *G. aculeatus* (ENSGACT00000010662).

3β-HSD yielded products of ~65 bp for both the forward and reverse primers, (expected length of 112 bp). Using BLAST, the degree of similarity of the forward and reverse primers to their mRNA sequences in *G. aculeatus* was acceptable at 65% and very strong at 97%, respectively. Only a forward primer sequence was generated for StAR of ~63 bp (expected to be 91 bp). Fortunately, this sequence had 97% alignment to the *G. aculeatus* StAR mRNA sequence, and >89% alignment with StAR mRNA sequences in other species, providing strong evidence that the primers were targeting the intended gene sequences. The RT-PCRs with the primers resulted in the appropriate product sizes for all primer pairs by gel electrophoresis. Thus, these results confirmed the primers were targeting the desired genes.

5.2.6.6 Real-time PCR: Optimization

A practice 96-well plate (MicroAmp Optical 96-well Reaction Plate, Applied Biosystems Inc.) was prepared with a cDNA sample at a concentration dilution of 1:10, run at either 5 or 10 μl. This plate was run to ensure an appropriate amount of cDNA was used, all primers functioned, and no primer-dimers interfered with the results. The plates were prepared as follows:

1. In the PCR Clean Room, each primer was diluted from 50 mM stock kept at -20°C to a concentration of 5 mM by adding 1 μl primer stock + 9 μl dH_20 in a new, labelled vial, and stored on ice.
2. Outside the Clean Room, a cDNA sample was diluted to 1:10 in sterile dH_20, to a final volume of 60 μl. The diluted sample was kept on ice and brought to the Clean Room.
3. In the Clean Room, four Master Mixes were prepared on ice under low light conditions, one for each primer set (StAR, 3β-HSD, β-Actin and Ubiquitin) according to the following two protocols:

a) Low cDNA: Prepared as Mastermix and added 20 μl to each sample	12.5 μl	SYBR Green PCR Master Mix (2X) (Applied Biosystems Inc.)
	1.0 μl	Forward Primer (5mM)
	1.0 μl	Reverse Primer (5mM)
	5.5 μl	dH_20 (autoclaved)
		+
	5 μl	cDNA samples (diluted 1:10)

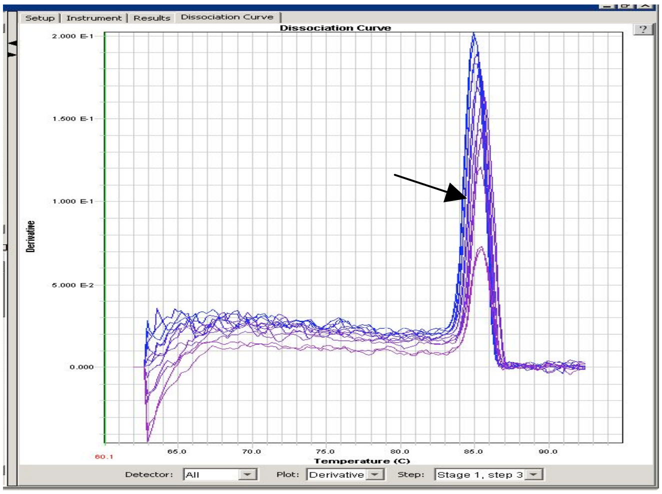
b) High cDNA: Prepared as Mastermix and added 15 μl to each sample	12.5 μl	SYBR Green PCR Master Mix (2X) (Applied Biosystems Inc.)
	1.0 μl	Forward Primer (5mM)
	1.0 μl	Reverse Primer (5mM)
	0.5 μl	dH_20 (autoclaved)
		+
	10 μl	cDNA samples (diluted 1:10)

4. In a sterile MicroAmp Optical 96-Well Reaction Plate (Applied Biosystems Inc.), the appropriate Master Mix was added to one side of each well, then the appropriate cDNA samples (1:10 dilution) were added to each well in either the 5 or 10 μl volumes. The plate was sealed with an adhesive cover and insulating pad (MicroAmp Optical Adhesive Film Kit, Applied Biosystems Inc.). The plate was kept in a darkened ice box. Within 15 minutes, it was spun to 600 RPM in a plate centrifuge and placed in the qRT-PCR machine for analysis.

The real-time PCR plates were amplified on the PTC-225 Pelter Thermal Cycler (MJ Research Inc.), detected with the ABI Prism 7900HT Sequence Detection System (Applied Biosystems Inc.) and then analysed by the SDS 2.1 computer program using the following protocol:

50°C for 2 min
95°C for 10 min
95°C for 15 seconds } Repeated for 40 cycles
60°C for 1 min

After the run, a dissociation (or melting) curve was conducted. This involved heating the plate to 95°C for 15 seconds at a 100% ramp rate, then to 60°C (15 seconds at a 100% ramp rate), and again 95°C (15 seconds at a 2% ramp rate). The dissociation curve measures fluorescence of each well (by a first-order derivative) as the temperature is slowly increased. When the temperature reaches the specific melting temperature of a product, the product is indicated by a peak. If several peaks at different temperatures are present, this indicates the presence of other products in the sample, such as primer-dimers and contamination products (eg. amplified bacterial DNA). An example of the results of an acceptable dissociation curve are shown in the figure below (Figure 5.6).



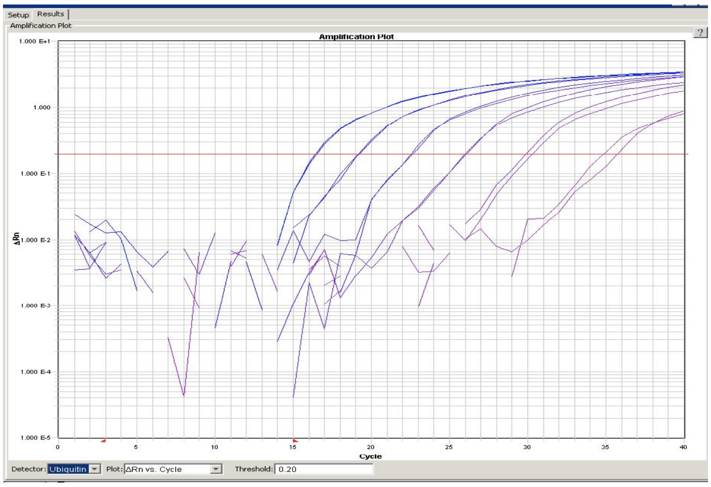
Detector: All
Plot: Derivative
Step: Stage 1, step 3

Figure 5.6. A photograph of the SDS 2.1 program's dissociation curve of Ubiquitin, plotting temperature by the derivative of fluorescence. All sample peaks are shown at a single melting temperature, and thus, a single product was present (arrow).

The practice plate ran without incident for all primer sets and all cDNA concentrations. The optimal conditions for qRT-PCR for all primer pairs was determined to be 5 μl of cDNA (diluted 1:10) run with 20 μl of Master Mix (with 5.5 μl dH_20), according to the same temperature protocol as outlined above.

5.2.6.7 Real-time PCR: Efficiency curves

A 96-well plate was run with a randomly selected cDNA sample to determine the efficiency of each primer pair. This was done so that the expression of the different genes could be compared directly within a sample by the Pfaffl method of quantification (Pfaffl 2001). In order to apply this method to the analysis of gene expression, the efficiency of each primer set first needed to be determined. Ideally, the efficiency of each primer pair is 2, so that every time 1 double-stranded cDNA fragment is replicated, 2 double-stranded copies result. However, in reality primers are not 100% efficient, and thus the number of copies is less than 2. Efficiency curves were determined by running serially diluted cDNA (1×10^{-6} to 1×10^{0}) with each primer set in a qRT-PCR (in duplicate). As stated previously, qRT-PCR measures the increase in fluorescence of the SYBR Green, which is dependent on the amount of double-stranded DNA, increasing with each cycle. When the normalized fluorescence (ΔRn) is plotted against the Cycle number, the results of a qRT-PCR appear as in Figure 5.7. Efficiency is calculated from a graph such as this, by first determining the average Ct of each cDNA dilution concentration. The Ct values are the cycle number at which the fluorescence meets a threshold, a value arbitrarily set by the computer program. Each Ct value is inversely correlated to the original amount of cDNA. For example, in Figure 5.7, the further the curves are to the right, the more concentrated the cDNA. Once the Ct values are known, they are plotted against their nominal dilution concentration, and a linear regression is drawn. The slope of this linear regression indicates the efficiency (E) according to the equation $E=10^{(-1/\text{slope})}$. Using this method, the efficiencies of the primers were as follows: Star 1.907, 3β-HSD 1.768, β-Actin 1.848, and Ubiquitin 1.853.



Detector: Ubiquitin Plot: ΔRn vs. Cycle Threshold: 0.20

Figure 5.7. Graph of the qRT-PCR run of Ubiquitin. Fluorescence (ΔRn) is plotted against cycle number. The red line indicates the threshold. The curves are the duplicated serial dilutions of cDNA diluted 0, 10, 100, 1000, 10,000, and 100,000 times.

5.2.6.8 Real-time PCR analysis of the male three-spined stickleback testes samples

Once the qRT-PCR protocol had been optimized, the actual samples for analysis were prepared in the 96-well plates in triplicate. Each 96-well plate was run with randomly selected samples and a negative control (blank). Outside the Clean Room, the cDNA samples were diluted 1:10 in 50 μl sterile tubes to yield a total volume of (5 μl/sample x (n samples + 2)). The diluted samples were kept on ice and brought to the Clean Room. The Master Mixes for the target genes 3β-HSD, StAR, and the housekeeping genes Ubiquitin and β-actin, were prepared under low light and added to the wells in 20 μl volumes. The cDNA samples were then added to the appropriate wells in 5 μl volumes. Once all components had been added, the plate was prepared and run according to the methods outlined in Section 5.2.6.6. Once the qRT-PCR run was complete, a dissociation curve was run for each plate to ensure no anomalies had occurred.

The Pfaffl method was used to analyse the results by comparing the mean Ct value of each sample with the following equation, where E_{target} is the efficiency of the target gene primers (StAR or 3β-HSD) and E_{ref} is the efficiency of the housekeeping gene primers (β-actin), and "average control" is the average Ct value of all of the samples from the Solvent Control group for that gene (target or reference) (Pfaffl 2001).

$$\text{Ratio} = \frac{E_{\text{target}}^{\Delta\text{Ct target (average control – sample)}}}{E_{\text{ref}}^{\Delta\text{Ct ref (average control – sample)}}}$$

5.2.7 *Statistical analysis*

One-way analysis of variance was used to analyse normally distributed data collected from these three-spined stickleback males including the analysis of plasma steroid hormone concentrations, nuptial colouration and spiggin concentration. Real-time PCR results were also analysed by ANOVA, with 3β-HSD and StAR analysed separately. Kruskal-Wallis one-way ANOVA on Ranks with Tukey Test was used to analyse the length and weights of the males in this experiment and the water chemistry results, as they were not normally distributed. Kaplan-Meier Survival Analysis Log-Rank was used to determine if there were any significant differences in the timing of nest-building.

5.3 Results

5.3.1 *Water chemistry*

All extracted water samples were analyzed in one GCMS run on April 1st, 2009, and again on April 6th, 2009. Initially, the samples were measured in chronological order, but were subsequently randomized to ensure any changes over time during the GCMS run did not affect the results. The results of both runs were very similar, and for the sake of simplicity, only the results of the April 6th run will be discussed.

The standard curve reported an $R^2 > 0.99$ for both DBP and BBP standards. The coefficient of variation between all DBP and BBP standards was under 20% for both runs, suggesting that the GCMS was reporting with both precision and accuracy. The 100% recovery value of the BBP internal standard was based on the mean of direct injections of the BBP standard at 27.12 ± 2.53 mg/L. Recovery was very high, with a mean of 95.65 ± 10.13% (SD).

The Solvent Control water appeared to be free from significant phthalate contamination. Three Solvent Control samples did have measurable concentrations of approximately 2.30 to 2.82 μg DBP/L on three separate days, but this was not measured in the duplicates collected on the same day. Ten water samples had DEHP contamination equivalent to approximately 2-4 μg/L (25% of all samples); these included four samples from the Solvent Control, two from the 50 μg DBP/L group, and four from the 100 μg DBP/L group. However, these DEHP peaks were only observed in one replicated sample on each sample day and no other occurrences of phthalate contamination were measured during the experiment. Overall, the measured concentrations of DBP were much lower than their nominal values (Figure 5.8).

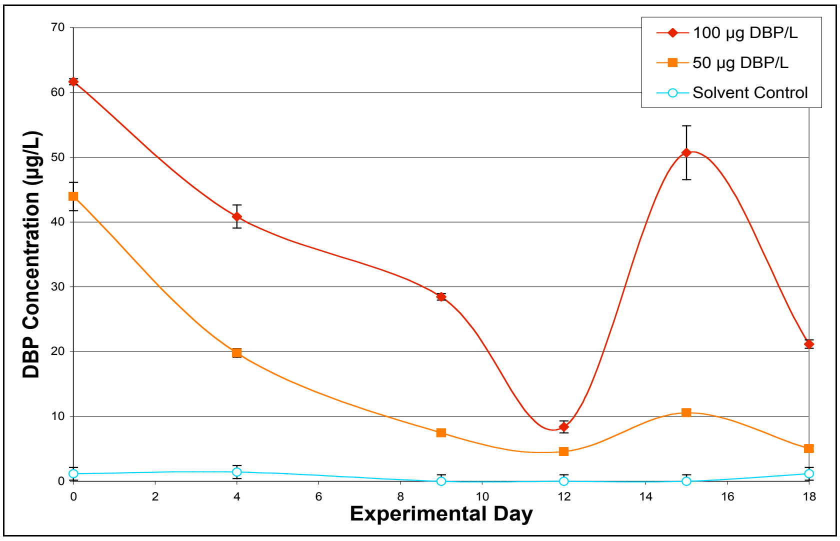


Figure 5.8. Concentrations of DBP (mean ± SD) in tank water over a 22 day period during the exposure of adult male three-spined sticklebacks in a nesting protocol. Concentrations are calculated by percent recovery of an internal standard.

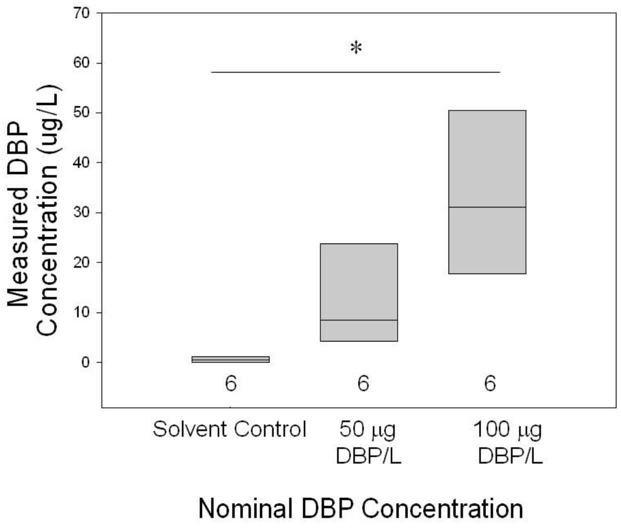


Figure 5.9. Box plot of the measured DBP concentrations in the water samples collected from the three treatments exposing adult male three-spined sticklebacks to DBP for 22 days.

The concentrations of DBP measured from the tank water samples appeared to span a range of concentrations. The highest exposure concentration was significantly different from the Solvent Control and all treatment groups had increasing DBP concentrations related to their nominal concentration ($P<0.05$, df. 2, ANOVA on Ranks). Unfortunately, there was also some overlap of DBP concentration between the 50 and 100 μg DBP/L groups (Figure 5.9), but this overlap did not occur at the same time (Figure 5.8). The mean concentrations of the 50 and 100 μg DBP/L tanks during the experiment were 15.23 ± 6.28 and 35.20 ± 8.03 μg DBP/L, respectively, but median concentrations were only 9.02 μg DBP/L (50 μg DBP/L tank), and 34.65 μg DBP/L (100 μg DBP/L tank). Thus, we concluded that the nominal concentrations were inaccurate. The 50 μg DBP/L group was therefore considered to be 15 μg DBP/L, and the 100 μg DBP/L group, 35 μg DBP/L.

5.3.2 *Length, weight, and gonadosomatic index*

In the 3 weeks in which the male three-spined sticklebacks were exposed to DBP no significant differences in weight, length, or gonadosomatic index developed ($P>0.05$, df. 2, ANOVA on Ranks).

5.3.3 *Plasma androgen concentrations*

The concentrations of 11-KT in the plasma of DBP-exposed males were not significantly different from those in the Solvent Control group, although plasma 11-KT concentrations in the fish exposed to 35 μg DBP/L appeared to be slightly higher ($P=0.372$, df. 23, ANOVA). However, plasma testosterone concentrations were observed to be higher in a concentration-dependent manner in the DBP-exposed fish compared those from the Solvent Control group. This apparent effect of DBP was significant when the 35 μg DBP/L group was compared to the Solvent Control group (P=0.007, df. 23, ANOVA) (Figure 5.10).

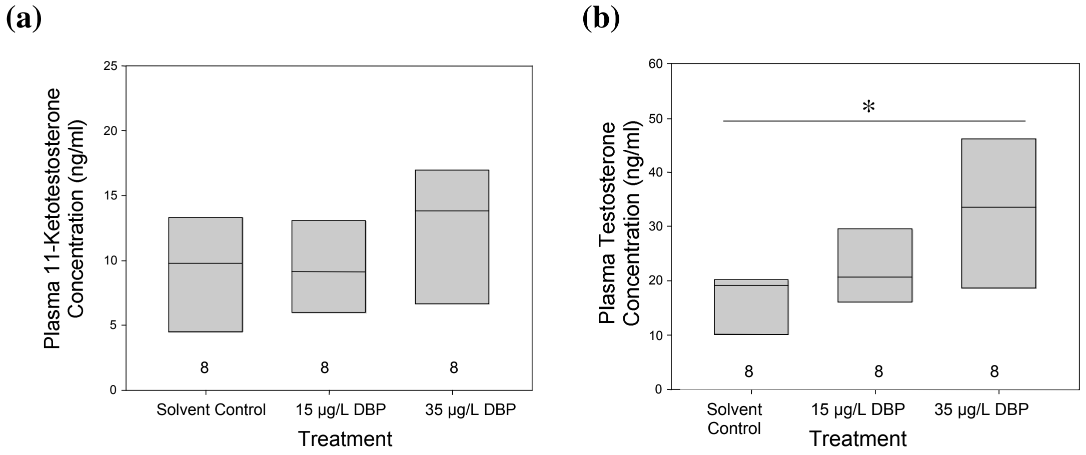


Figure 5.10. Box plots (25th, median, and 75th percentiles) of the plasma concentrations of (a) 11-KT, and (b) testosterone in male three-spined sticklebacks exposed to various concentrations of DBP in a 22-day nesting study (n=8, * $P<0.05$ compared to Solvent Control group).

5.3.4 *Spiggin*

Interestingly, spiggin concentrations followed the opposite response to DBP-exposure compared to the plasma androgen concentrations. Overall, there seemed to be an anti-androgenic response; concentrations of spiggin decreased with increasing DBP concentration. There was one outlier in the spiggin data: a very low spiggin concentration in one of the Solvent Control males. This was considered to be an outlier because it was more than two standard deviations from the mean. Further, it was both the first kidney sample to be collected by the laboratory technician and the first sample in the spiggin ELISA run. Thus, it is possible that either the poor sample collection or technical error at the beginning of the ELISA may have resulted in its low spiggin concentration. Once omitted, the results showed a strong negative correlation between spiggin concentration and DBP treatment, significant at the highest DBP concentration (P=0.011, df, 22, ANOVA) (Figure 5.11).

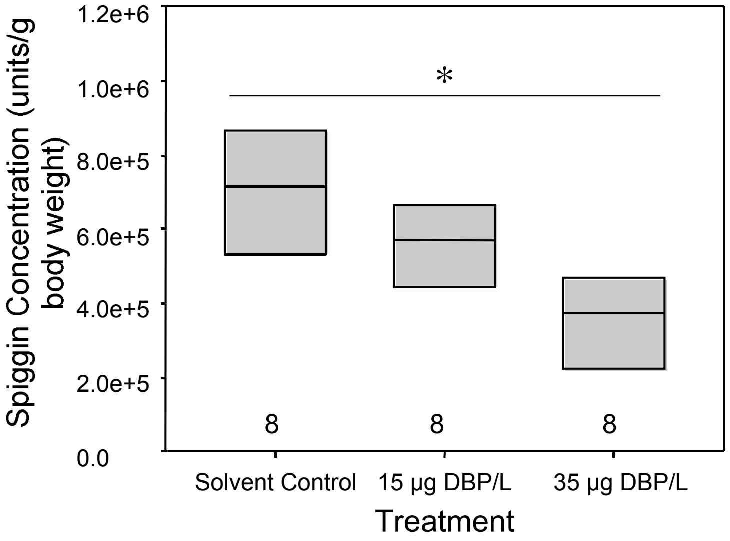


Figure 5.11. Box plot of spiggin concentrations in male three-spined sticklebacks exposed to various concentrations of DBP for 22 days (n=8, *P<0.05 compared to the Solvent Control group).

5.3.5 *Nuptial Colouration*

The degree of redness of the throat of nuptially coloured males was not significantly different between treatments (P=0.400, df. 22, ANOVA) (Figure 5.12). Further, throat redness did not appear to be correlated to spiggin concentration (R^2=0.0231) (Figure 5.13), plasma 11-KT (R^2=0.0138), or testosterone (R^2=0.0013) (P>0.05, df. 23, Pearson Product Moment Correlations).

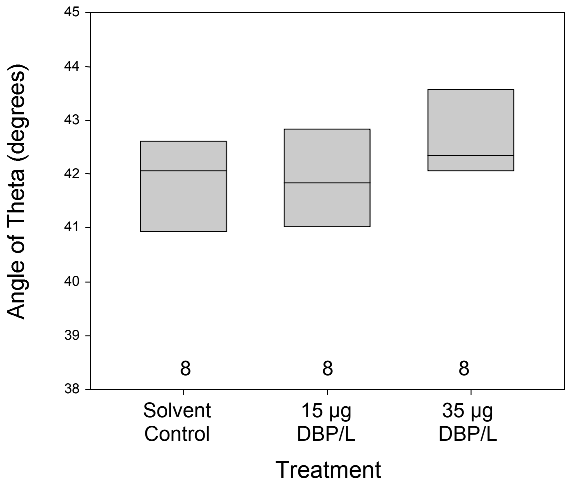


Figure 5.12. Box plot of the angle of theta from the red axis of the throat areas of mature three-spined stickleback male, following exposure to various concentrations of DBP for 22 days. (The smaller the angle, the redder the throat).

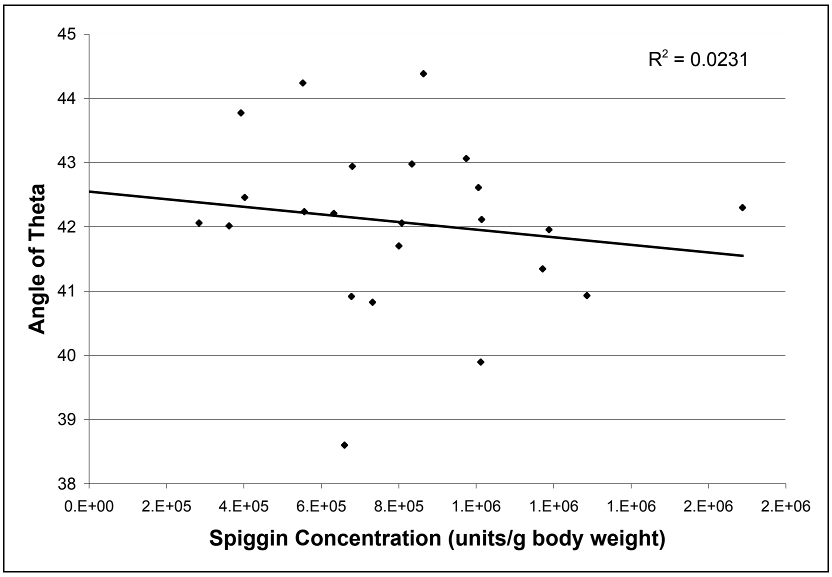


Figure 5.13. Plot of the angle of throat redness (theta) compared to spiggin concentration in three-spined stickleback males exposed to various concentrations of DBP for 22 days.

5.3.6 *Nest-Building Behaviour*

Nest building appeared to be slightly delayed in the highest DBP treatment group. All eight males in the Solvent Control had completed their nests by Day 15. By contrast, three males in the highest treatment concentration of DBP (35 μg DBP/L) did not build nests until Day 22, the last day of the experiment. However, there were no significant differences between the rates of nest-building in males exposed to DBP compared to rates of the Solvent Control group. Five males from both the Solvent Control and 15 μg DBP/L groups, and four from the 35 μg DBP/L treatment group, had all built nests one day following the addition of nesting material (P=0.355, df. 2, Kaplan-Meier Survival Analysis). By the end of the experiment, all but one male in the 15 μg DBP/L treatment had built and maintained their nests (Figure 5.14).

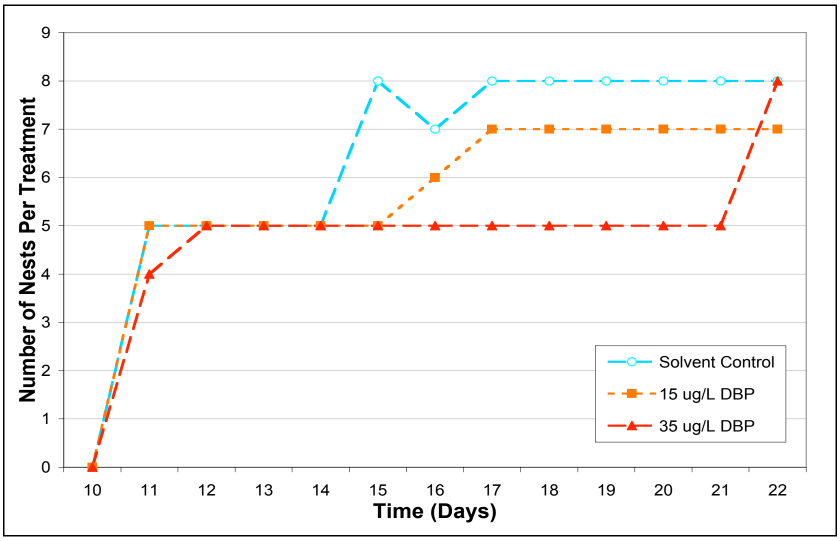


Figure 5.14. Graph of the cumulative number of nests in each treatment group over time, during an experiment involving exposing adult male three-spined sticklebacks to various concentrations of DBP for 22 days (n=8).

5.3.7 *Real-time RT-PCR*

The expression of both housekeeping genes, Ubiquitin and β-actin, was not statistically significant when comparing Ct values across treatment groups (P= 0.156 and P=0.537, respectively, df. 23, ANOVA), which was a positive finding with regard to their ability to act as housekeeping genes. However, there did appear to be a dose-related increase in the expression of Ubiquitin with DBP-treatment and a high risk of Type II Error. Thus, the results presented herein were normalised against the expression of the β-actin gene only.

The expression of the steroidogenic genes, StAR and 3β-HSD, did not appear to be affected by DBP exposure. There were no significant differences between the expression of either gene in the testes of the males exposed to any concentration of DBP compared to controls (P = 0.915 for StAR, and P=0.975 for 3β-HSD) (both df. 23, ANOVA) (Figure 5.15).

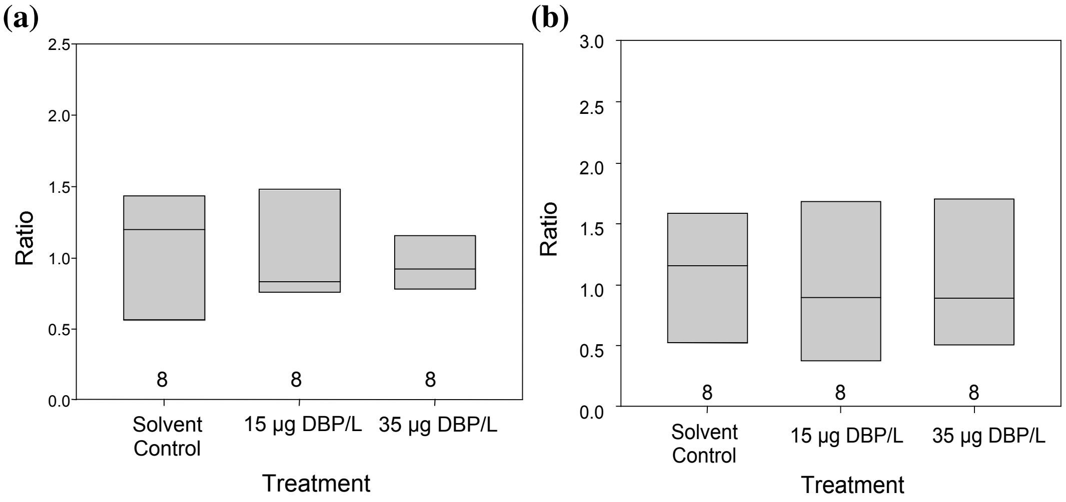


Figure 5.15. Box plot of the expression of the genes (a) StAR and (b) 3β-HSD in the testes of the male three-spined stickleback exposed to DBP. The expression ratios have been calculated according to the Pfaffl method, normalised to the expression level of β-actin.

5.4 Discussion

5.4.1 *The use of wild fish for experimentation*

When studying low-level effects of chemicals on fish behaviour and physiology, it is important that any confounding factors be minimized. The use of wild fish in this experiment was a potential risk to the interpretation of results, due to the differences in age and life history of the fish used. For example, it is possible that some, or all, of the fish used in the current experiment had been exposed to contaminants, including endocrine disruptors, during critical stages in development that may have later affected their behaviour and/or physiology.

However, since the fish spent 201 days in the laboratory prior to their use in any experiments, we must assume that the majority of any chemicals bioconcentrated in tissues while the fish were in the wild would likely have been metabolised and excreted by the beginning of the experiment. This, of course, does not negate the possibility that other endocrine disruptors may have caused permanent effects, which could have confounded the results. However, the random distribution of the experimental males should have mitigated any potential confounding factors between individuals. It must then be assumed that any differences between the treatments are attributable only to the effects of DBP.

5.4.2 *Glugea anomal infection and mebendazole exposure*

Due to the *Glugea anomala* infection, it was seen as a necessary precaution to treat all wild-caught fish with mebendazole to prevent further mortality and infection of the wild-caught three-spined sticklebacks, and also prevent the spread of the infection to other stickleback populations in the laboratory. Unfortunately, the *in vivo* androgenic activity of mebendazole presented a potentially confounding effect on the results of the anti-androgen DBP. While there is very little published data on the metabolism of this drug or any of its close relatives, such as albendazole, we have assumed that the 100-day period following treatment was sufficient for the fish to fully metabolise and excrete this drug. On all three occasions, 22.5 mg total of mebendazole was added to the Male stock tank containing 18 fish. If the fish absorbed 100% of the mebendazole, then each fish took up a total of 3.75 mg mebendazole, which is equivalent to an androgenic exposure of 3.75 μg DHT per fish. Considering the potency of DHT, this is a fairly high dose of androgen. However, there are several factors which negate any potentially confounding effects of mebendazole on the results of this study. Firstly, all males were exposed to the same concentrations of mebendazole, since they were pooled in the same tank. This would invalidate any differing effects of mebendazole between the Solvent Control and DBP-exposed groups of males. Secondly, the fish had 104 days following the last mebendazole exposure in which to metabolise and excrete any of the remaining drug. Mebendazole is fairly water-soluble and its rapid metabolism has been noted in humans, occurring in a matter of hours (Braithwaite *et al.* 1982). Thirdly, the period required for male kidney hypertrophy to regress from its sexual stage to quiescence is stated as, at minimum, one month and, at maximum, three months (Katsiadaki *et al.* 2007). Thus, even if the mebendazole had induced spiggin production in the males, the three months prior to the experimental period would have allowed enough time for the kidney of any affected males to regress into quiescence. Thus, we conclude that it is highly unlikely that the treatment of the males with mebendazole interfered with the results from this experiment.

It is also possible that the *Glugea anomala* infection was not fully treated by the administration of mebendazole. This is unlikely, as further pathological analysis of the remaining three-spined sticklebacks showed no suggestions that the infection had persisted. *Glugea anomala* has been found to interfere significantly with metabolic rate and shoaling behaviour of three-spined sticklebacks (Ward *et al.* 2005), but this parasite has not been associated with changes in any reproductive behaviours. However, infections with other parasites, such as plerocercoids of the cestode *Schistocephalus spp.,* have been found to significantly reduce the expression of secondary sexual characteristics, nest building, reproductive behaviours and even spiggin concentrations in male three-spined sticklebacks (Rushbrook and Barber 2006; Rushbrook *et al.* 2007). Thus, it was important that *Glugea anomala* was eradicated to ensure it did not interfere with the males in the experiment. However, in the event that this parasite had persisted after the mebendazole treatments, the results of the current experiment were likely not skewed in any treatment group compared to another because, again, all males were treated with mebendazole in the same tank and randomly distributed throughout the experimental system.

5.4.3 *Analytical Chemistry*

The administration of DBP through the flow-through system did not appear to be consistent during the exposure period (Figures 5.8 and 5.9). However, the analytical results generally suggest that the fish in the (nominal) 50 and 100 μg DBP/L tanks were exposed to a range of concentrations of DBP throughout the experiment, which was significantly different from the Solvent Control in the case of the highest DBP treatment group (35 μg DBP/L).

While DBP has been previously maintained at levels closer to the nominal tank water concentrations in our laboratory (Chapter 4), it appears that phthalates are particularly difficult to administer via water at stable concentrations. Similar difficulties have been reported in other laboratories using another phthalate ester, BBP (Dr. Eduarda Santos, Exeter University, personal communication). The variable concentrations of DBP in the tank water might also be related to the accumulation of bacterial growth noted during the course of the experiment, which may explain the initial decline over the first 12 days. While this had not previously been found to affect the DBP concentrations (Chapter 4), in this experiment the tanks were first cleaned on Day 12, after the water samples had been collected and extracted. As shown in Figure 5.8, the concentrations of DBP increased in the following days.

While the measured concentrations of DBP were not collected from water sampled directly from the fish tanks, we assume they are accurate representations of the actual concentrations in the fish tanks. If we assume the DBP was mixed homogeneously in the distribution trays, there is no reason to believe that the Analytical Chemistry Tank would be receiving more or less DBP than the other fish tanks at any given time. In future, it might be wiser to sample water from the outflow of the fish tanks, to minimize disturbance of the males while measuring the concentrations directly. However, we feel the results presented there are highly likely to be accurate representations of the concentrations of DBP the fish were exposed to.

5.4.4 *Physiological effects of DBP in the adult male three-spined stickleback*

As expected, DBP exposure did not have any effect on fish length, weight, or gonadosomatic index. This supports the findings of the previous experiments, in which the lengths, weights, and gonadosomatic indices of both the fathead minnows and three-spined sticklebacks exposed to various concentrations of DBP were unaffected by DBP-exposure.

The results of the real-time PCR analysis, hormone concentrations, and spiggin assay suggest a somewhat conflicting picture. Contrary to our expectations, DBP-exposure was associated with increases in the plasma concentrations of 11-KT and testosterone, which was significant for testosterone only (Figure 5.10). However, no significant changes to steroidogenic gene expression were measured (Figure 5.15).

It is unclear whether or not the lack of any effect on gene expression was a true reflection of the results, or because it was overshadowed by confounding factors, such as age, GSI, or gonadal stage. Previous studies with fathead minnows found that the expression of both StAR and 3β-HSD were significantly higher in older males. GSI in males was also found to significantly affect the expression of StAR, while the histological stage of the gonads was found to significantly increase the expression of 3β-HSD (Villeneuve *et al.* 2007). To further confound matters, even when these factors were taken into account, the exposure of fathead minnows to the anti-androgen ketoconazole (4, 25, 100, and 400 μg/L), was observed to result in significantly increased expression of StAR in the testes of male fathead minnows exposed at concentrations of 6 and 400 μg/L, although this was not concentration-related (Villeneuve *et al.* 2007). Thus, the expression of steroidogenic genes in fish may be easily affected by other factors, and further, its response to anti-androgenic chemicals, even when they are known to directly interfere with steroidogenesis, remains poorly understood.

However, in accordance with our assumptions, spiggin concentrations were strongly reduced, an effect that was significant at the highest DBP concentration

(Figure 5.11). Spiggin is a sensitive biomarker to androgenic disruption, able to undergo 100,000-fold increases in response to androgen concentrations (Katsiadaki *et al.* 2002). Spiggin can be measured within 3 weeks of induction *in vivo,* and takes only 24-48 hours to be produced and accurately measured in *in vitro* primary kidney cultures (Bjorkblom *et al.* 2007; Jolly *et al.* 2006). The half-life of spiggin in the three-spined stickleback is unknown, but it is estimated that it requires 1 to 3 months for it to be degraded to background levels in sexually mature males (Katsiadaki *et al.* 2007). Thus, we can assume that while spiggin production is rapidly induced by androgens, it is also a complex protein, requiring weeks to months to be broken down by the body. In other words, spiggin appears to be a longer-term biomarker than hormone concentrations. Thus, it may give a more rounded picture of an endocrine response than plasma hormone concentrations, which can change rapidly and are fairly variable.

Several studies have shown that exposure of three-spined sticklebacks to androgens induces spiggin production (Borg 1981; Jolly *et al.* 2009; Katsiadaki *et al.* 2007; Mayer *et al.* 1990). Similarly, several studies have shown that it is consistently reduced following anti-androgenic endocrine disruption in both *in vitro* and *in vivo* assays (Jolly et al. 2009; Katsiadaki et al. 2006; Sebire et al. 2008). However, there is currently a paucity of research on how spiggin concentrations change in relation to plasma androgen concentrations over time. Consequently, it is unclear whether or not these parameters are positively-correlated to each other over time, and if so how closely. For example, it remains unknown whether spiggin production is maintained by androgens or simply triggered by them.

In contrast to spiggin, plasma hormone concentrations may fluctuate more rapidly from one day to the next, since their half-life is much shorter. This fluctuation in steroid hormones can be more easily observed by measuring cortisol during a stress response in fish. When rainbow trout were placed in confined tanks for one hour (at time 0) the plasma cortisol concentrations rose from 0 to 80 ng/ml serum in only half an hour. Once the stressful conditions were removed, the cortisol concentrations took only 6 hours to fall back to background concentrations (Pickering *et al.* 1991). The concentrations of sex hormones can also be highly variable over time. For example, the concentrations of the sex hormones in female rainbow trout change drastically over a matter of days, based on the stage they have reached in their ovulation cycle (Scott *et al.* 1983). In male three-spined sticklebacks, fluctuations in response to the timing of the spawning cycle have also been observed. Mayer *et al.* (1990) measured plasma androgen concentrations in male three-spined sticklebacks over the course of a year and observed a large increase in the plasma 11-KT concentration in response to the onset of the breeding condition. However, this fluctuation was not as drastic in the plasma testosterone concentrations and also appeared to fluctuate over a period of months rather than days.

Overall, it remains unclear as to why the plasma androgen concentrations in the three-spined sticklebacks exposed to DBP in the current study were found to increase (significantly, in the case of testosterone), while spiggin concentrations were significantly decreased. As stated, this may be due to the unpredictable responses of plasma androgens to phthalates, to anti-androgens in general, to feedback responses, and/or to the inherent fluctuation of plasma concentrations both in general and in response to spawning stage.

As I have discussed previously, the mechanism of action of phthalates as anti-androgens is poorly understood, as is their effect on plasma hormone concentrations. On the one hand, several studies report elevated plasma androgen concentrations and increases in steroidogenic gene expression following phthalate exposure in rats (Akingbemi *et al.* 2004; Borch *et al.* 2006; Culty *et al.* 2008; Lahousse *et al.* 2006; Lee *et al.* 2009; Ryu *et al.* 2007). On the other hand, a prevailing majority of studies report the opposite (Andrade *et al.* 2006; Barlow *et al.* 2003; Borch *et al.* 2004; Lehmann *et al.* 2004; Lin *et al.* 2008; Shultz *et al.* 2001; Thompson *et al.* 2004). While reports of changes in plasma androgen concentrations and expression of steroidogenic genes have been variable, what remains clear is that endpoints dependent on androgen concentrations are substantially more consistent in their response to anti-androgenic chemicals than the androgen concentrations themselves. In male rats exposed to phthalates *in utero,* reduced anogenital distance, nipple retention and agenesis of the Wolffian ducts are repeatedly and consistently observed (Andrade *et al.* 2006; Gray *et al.* 2000; Mylchreest *et al.* 1999; Mylchreest *et al.* 2002; Mylchreest *et al.* 2000). While these effects do not occur with 100% frequency, their dependence on androgens supports the suggestion that phthalates cause anti-androgenic effects *in vivo*. In a similar manner, the result of reduced spiggin concentrations in DBP-exposed fish in this experiment provides strong support for our hypothesis that DBP acts as an anti-androgen in fish, despite increases in plasma testosterone concentrations and the absence of changes in steroidogenic gene expression. This is, of course, countered by a lack of effect on other androgen-dependent parameters in our study, such as nuptial colouration and nest-building (Figures 5.12 and 5.14). However, such changes are probably not nearly as sensitive as spiggin. For example, as discussed previously, nuptial colouration may not be sensitive enough under laboratory conditions to “honestly” indicate differences between males (Barber *et al.* 2000; Candolin 2000).

In some respects, the fact that the actual concentrations of DBP measured during this experiment were approximately one third and one sixth of their nominal concentrations, suggests that phthalates may be more potent anti-androgens in fish than expected. Perhaps at higher concentrations of DBP, spiggin concentration might be further reduced, and nest-building and nuptial colouration, as well as gene expression and plasma androgen concentrations might be reduced as well. This suggestion is supported by the results of mammalian studies, in which very high doses (>500 mg/kg/day) are required to elicit measurable anti-androgenic effects (Akingbemi *et al.* 2004; Borch *et al.* 2004; Lin *et al.* 2008).

When we compare the findings of the effects of DBP on spiggin concentrations in the adult male three-spined sticklebacks from this experiment, to the effects on the sticklebacks exposed during early life (Chapter 4), they are again contradictory. However, in the current experiment, the males were sampled immediately after they were exposed to DBP, and not 4 months after DBP-exposure had ceased. This provides strong evidence that DBP causes reduced spiggin concentrations in adult male three-spined stickleback while they are being exposed to phthalates.

Despite the effects DBP appeared to have on plasma androgen concentrations and spiggin, this failed to significantly affect nest-building behaviour. A few males exposed to the highest concentration of DBP did take longer to build their nests. However, in general, all of the males across all groups built nests at a similar rate and were successful in maintaining them (except one male in the 15 μg DBP/L group). This suggests that phthalate exposure at concentrations of 15 and 35 μg/L does not significantly impede the nest building behaviours of the male three-spined stickleback.

Finally, it remains unclear whether or not adult male three-spined sticklebacks exposed to DBP would have been able to spawn successfully, and also if they would have been capable of competing for females. Considering the findings of the previous early life-stage exposure (Chapter 4), combined with the ability of these males to build nests, DBP exposure at such low concentrations may not be potent enough to significantly affect reproduction in adult male three-spined sticklebacks. However, considering that spiggin was significantly reduced in males exposed to these low concentrations, it is possible that marginally higher concentrations may interfere with ability of these fish to reproduce completely. For example, DBP at higher concentrations may reduce spiggin production to such an extent that males may not be able to build nests and, ultimately, to spawn. That possibility merits investigation.

5.5 Conclusions

DBP did not have any effect on nest-building behaviour, on the redness of nuptial colouration, and did not inhibit the expression of two steroidogenic genes in the testes at concentrations of 15 and 35 μg/L.

However, DBP significantly increased plasma testosterone concentrations, and significantly reduced spiggin concentrations in exposed three-spined sticklebacks in a concentration-related manner after 22 days. These findings, combined with the results of lower-than-expected aqueous DBP concentrations, suggests that DBP does have the ability to act as an endocrine disruptor in sexually mature three-spined sticklebacks at concentrations that have been measured in the aquatic environment. More research is needed to determine if DBP, at slightly higher concentrations than those I tested, is able to significantly inhibit the behaviour and physiology of male three-spined sticklebacks, ultimately interfering with their ability to reproduce.

Chapter 6 Summary and Conclusions

The work presented herein addressed the question of whether or not phthalate esters are able to elicit anti-androgenic endocrine disruption in fish. While the results were not completely clear-cut, the repeated findings of significantly altered plasma hormone concentrations in both the fathead minnow and three-spined stickleback, as well as significantly altered spiggin concentrations in some of the three-spined sticklebacks, suggest that environmentally-relevant concentrations of phthalates may be able to interfere with the normal endocrine function of fish.

The work also presented several opportunities to develop my research skills; many obstacles had to be overcome in order to properly address the hypothesis. The first challenge was the establishment of accurate and robust analytical chemistry techniques for the measurement of DBP in the tank water. This proved to be much more difficult than anticipated, but demonstrated the importance of validated analytical techniques in fish exposure studies. Without water chemistry data, the results of experiments investigating the effects of low concentrations of chemicals on fish are simply unreliable. It is very easy to either report biological effects at concentrations of chemicals that are inaccurate, or to report the lack of an effect in fish to a chemical that is, in fact, absent. This is perhaps why the second challenge, the establishment of indirect evidence of the uptake of DBP by fish (Chapter 3 Sections 3.6 and 3.8), was so important. While the ability of fish to bioconcentrate DBP has previously been documented (Mayer and Sanders 1973; Staples *et al*. 1997; Vethaak *et al*. 2005), the two experiments I conducted suggested that DBP is absorbed very rapidly from the water in a flow-through system. This underlined the susceptibility of fish to phthalates in the wild, because the concentrations of phthalates in the aquatic environment may be highly variable, both temporally and in concentration. Hence, it appears that even infrequent inputs of phthalate esters to surface waters may pose significant threats to fish, due to the evidence presented: that they appear to absorb DBP rapidly and to such an extent that its concentration is significantly reduced in the tank water within hours.

Finally, this work underlined the importance of selecting the most sensitive and appropriate species for the investigation of an endocrine disrupting chemical. While there are several benefits to using the fathead minnow in endocrine disruption experiments, the decision to use the three-spined stickleback proved to be highly beneficial. This is mainly due to the ability of the three-spined stickleback to produce spiggin. The analysis of spiggin concentration proved to be very important to this work, because it is a very sensitive androgen-dependent biomarker. Since the mechanism of action of phthalates has yet to be determined, it was imperative that a robust biomarker for anti-androgenic activity was established, especially considering that the effects of phthalates on plasma androgen concentrations in both fish and mammals are inconsistent (Akingbemi *et al.* 2004; Akingbemi *et al.* 2001; Borch *et al.* 2006; Culty *et al.* 2008; Han *et al.* 2009; Higuchi *et al.* 2003; Lee *et al.* 2009; Lin *et al.* 2008).

Overall, the results of this work suggest that phthalate esters are able to affect fish populations at concentrations that can be found in the environment (Chapter 1, Table 1.1). In several of the experiments, DBP significantly altered the plasma androgen concentrations in the males of both species, and the spiggin concentrations in the male three-spined sticklebacks. However, despite the repetition of several experiments, such as the nesting studies (Chapter 4), these effects occurred inconsistently. It remains unclear as to why this was the case. Perhaps, as stated previously, this is due to the low concentrations of phthalates used, to the differences in the timing of exposure and when the fish were sampled, the lower-than-desired sample sizes, or perhaps due to the inconsistency of the response of plasma androgen concentrations to phthalates in general. It is also possible that some of these significant findings were the result of Type I Error.

The most striking result of this work as a whole, was the significantly lower spiggin concentrations in the three-spined stickleback males exposed as adults to 35 μg DBP/L after only 22 days (P=0.011; Chapter 5). This finding presents a real cause for concern over the effects of phthalates on fish populations, since these fish were fully mature when they were exposed, it was a short-term exposure, and they were exposed to relatively low concentrations of DBP. Unlike the more variable spiggin concentrations measured in the three-spined stickleback males that had been exposed in early-life (Chapter 4, Section 4.3), these adult males were examined immediately following DBP-exposure and thus, the effect of DBP on spiggin concentration may be a consequence of the DBP still being present in the fish tissues.

In general, while DBP did have a marked effect in this regard, it appeared to be rather benign in its ability to effect changes in the gonadal histology, secondary sexual characteristics, reproductive behaviours, and fecundity in both species. It is possible that a lack of significant effects on these parameters was due to the low potency of DBP as an anti-androgen. As stated previously, in mammals it is well documented that doses $\geq$500 mg/kg/day during development are generally required to elicit measurable changes in the offspring. Again, while it is impossible to compare the dietary exposure concentrations of rats to aqueous exposure concentrations in fish, it is still possible that concentrations of $\leq$200 μg DBP/L may have been too low to have affected the fish in this work. Higher concentrations, although less environmentally-relevant, may have caused anti-androgenic effects that would have been marked enough to be consistently observed.

It is also possible that the lack of sensitivity of several of the assays may have hindered our investigation. For example, it would have been ideal to use several immunohistochemical stains for the histological analysis of specific cells in the gonads, because doing so may have demonstrated subtle effects of DBP that were not visible using the standard histological approach. The measurement of testicular androgens (both *in situ* and *ex vivo* production) would also likely have been a more robust biomarker than the measurement of plasma androgen concentrations (Akingbemi *et al.* 2004; Akingbemi *et al.* 2001; Martinovic *et al.* 2008). A pair-breeding study would likely have been much more appropriate for the analysis of the effects of DBP on fecundity and secondary sexual characteristics in the fathead minnows (Ankley *et al.* 2001; Harries *et al.* 2000). Finally, the conditions under which the three-spined sticklebacks were kept may have been unsuitable for the accurate analysis of the redness of the nuptial colouration (Barber *et al.* 2000; Candolin 2000).

In future work, it would be interesting to pursue investigations into the effects of phthalate exposure on fish, especially regarding effects on:

- Leydig cell aggregation in the male testis, and oocyte atresia in the female ovary.
- Reproduction, including the timing of nest-building and the effects on fecundity.
- Testicular testosterone production *ex vivo* and concentration *in situ*.
- The ability of phthalate-exposed males to compete with unexposed males for mates.

- The effects of higher concentrations of phthalate on spiggin concentration, and whether such phthalate concentrations also affect secondary sexual characteristics and behaviours.

However, perhaps the most wanting area for phthalate-based research is the determination of the mechanism of action of phthalates. Only once this is established can appropriate experiments be designed that will help us to fully understand the effects of these weak anti-androgens *in vivo*.

Overall, the question all ecotoxicologists aim to answer is whether or not the chemicals identified in the environment are able to induce physiological responses in animals at the concentrations normally present in the environment. However, the ultimate question to be answered is whether or not such changes cause detrimental effects at the population, community, and/or ecosystem levels. Overall, the results presented in this work provide a small piece of the puzzle regarding the former question, but we have yet to answer the latter.

Laboratory experiments represent the effects of a chemical only within a very narrow context. Fish in the wild are not usually exposed to a single chemical at a consistent concentration in a non-competitive, resource-rich environment. In reality, phthalate esters make up only a small proportion of the endocrine disruptors (anti-androgenic or otherwise) present in surface waters (Vethaak *et al.* 2005). It is now thought that the widespread endocrine disruption affecting fish is due, not only to oestrogenic chemicals, but also to the plethora of anti-androgens present (Jobling *et al.* 2009). Since phthalates are such widely used chemicals, they should be considered with an appropriate degree of concern. However, this must be tempered with the fact that they are much less potent than other anti-androgens present in the environment, such as vinclozolin, flutamide, and DDE (Dargnat *et al*. 2009; Fatoki and Vernon 1990; Gray *et al*. 1999; Jolly *et al*. 2009; Kolpin *et al*. 2002; Peijnenburg and Struijs 2006; Urbatzka 2007; Valsamaki *et al*. 2007). It would be ideal to test the effects of phthalate esters on fish in a more "realistic" context. This would involve phthalate-exposure as part of a mixture of anti-androgens, possibly with various mechanisms of action, and also with a mixture of endocrine disruptors with other effects (eg. oestrogenic and androgenic antagonists and agonists) to determine whether or not additive effects may be occurring and the severity of such effects, since it is now held that synergistic effects do not generally occur (Sumpter and Johnson, 2008).

Regardless, the main conclusion of this study is that phthalate esters were sometimes able to induce significant changes to plasma androgen concentrations in the male fish of both species studied, and spiggin concentrations in the male three-spined stickleback. However, it appears that phthalate esters do not currently pose a significant risk to fish in the wild. Environmentally-relevant concentrations of DBP were unable to disrupt the normal sexual development and reproduction in either the fathead minnow or the three-spined stickleback.

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